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Assessment of Toll-like Receptor 4, Glycogen Synthase Kinase-3 Beta, and Oxidative Stress in Parkinsonism Mice Model Treated with Metformin

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ABSTRACT

Background: Parkinson's disease is a progressive neurological disorder that affects the substantia nigra, a brain region responsible for controlling body movement. In Parkinson's disease, the death of dopaminergic neurons is thought to result, at least in part, from a neuroinflammatory state that originates from the activation of toll-like receptor-4 (TLR4) and glycogen synthase kinase-3 beta (GSK3β), among other contributing mechanisms.
Objectives: To assess the neuroprotective role of metformin, alone or in combination with levodopa/carbidopa, against rotenone-induced Parkinsonism in male albino mice.
Materials and methods: Thirty healthy male albino mice weighing 25-30 grams were randomly assigned to six experimental groups (n = 5 per group). The total duration of the experiment was 47 days. The neuroprotective and therapeutic effects were evaluated by measuring serum levels of GSK3β, malondialdehyde (MDA), and superoxide dismutase (SOD) using enzyme-linked immunosorbent assay kits. Additionally, quantitative real-time polymerase chain reaction was performed to evaluate the messenger ribonucleic acid expression of TLR4 specifically in the substantia nigra region.
Results: Rotenone administration led to a significant elevation in GSK3β and MDA levels, a reduction in SOD activity, and upregulation of TLR4 expression in the substantia nigra, indicating its strong neurotoxic effect. Preventive administration of metformin effectively restored these molecular and oxidative stress markers to normal levels, demonstrating a clear protective role. Moreover, therapeutic intervention with metformin, whether administered alone or in combination with levodopa/carbidopa, significantly improved the biochemical and inflammatory profiles associated with rotenone exposure.
Conclusion: Metformin showed neuroprotective activity in the rotenone-induced model, potentially by modulating oxidative stress and inflammation.
Keywords: Metformin; Parkinson's disease; Rotenone; Toll-like Receptor 4; Glycogen Synthase Kinase-3 Beta; Oxidative stress.

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INTRODUCTION

Parkinson's disease (PD) is a long-term and progressively worsening disorder of the nervous system, predominantly affecting movement control. Although its characteristic motor symptoms, such as resting tremors, rigidity, and slowed movements, are well recognized, non-motor manifestations like cognitive deficits, sleep disturbances, and mood disorders often precede these motor signs by many years [1]. The distribution and prevalence of PD vary globally due to demographic factors including age, sex, ethnicity, and healthcare access, with recent data indicating a moderate burden of disease in Iraq [2, 3].

PD's neuropathogenesis is complex, multifaceted, and comprises several interconnected pathways. Numerous lines of evidence have demonstrated the crucial role that neuroinflammation and oxidative stress play in the development of PD [4]. Specifically, toll-like receptor-4 (TLR4), known for its role in immune response activating, has been implicated in exacerbating inflammation within the brain, potentially accel-

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erating dopaminergic neuron loss. Targeting TLR4-mediated pathways may therefore offer new therapeutic possibilities [5].

Increased malondialdehyde (MDA) concentrations reflect heightened free radical activity and lipid membrane damage, a well-established marker of oxidative damage. A decrease in superoxide dismutase (SOD) activity is typically observed in oxidative stress, as this antioxidant enzyme reflects the cellular defense mechanisms against oxidative damage [6]. Additionally, glycogen synthase kinase-3 beta (GSK3β) is implicated in nearly all aspects of brain activity, including synapse development, neuroinflammation, neuronal morphology, and neurological diseases [7]. Given the involvement of these molecular pathways in PD, there is a growing need for therapeutic approaches that target the underlying mechanisms. Currently, available treatments focus primarily on symptom relief, with levodopa (L-DOPA) and related agents alleviating motor impairments without halting disease progression [8].

Metformin (MET), traditionally used for managing type 2 diabetes mellitus, has emerged as a potential neuroprotective agent due to its ability to activate AMP-activated protein kinase (AMPK), reduce oxidative stress, and modulate inflammatory responses [9, 10]. A recent study further supports this potential, demonstrating that MET alleviated rotenone-induced parkinsonism-like symptoms and improved behavioral and biochemical outcomes in a mouse model [11].

To the best of our knowledge, this study is among the first to explore the involvement of TLR4 signaling in the neuroprotective actions of MET within a rotenone-induced PD model. Therefore, this study aimed to evaluate the potential protective effects of MET, either alone or in combination with L-DOPA/carbidopa, against rotenone-induced neurotoxicity, with a specific focus on its modulation of molecular and oxidative stress markers such as TLR4, GSK-3β, SOD, and MDA.

MATERIALS AND METHODS

Materials

Pure rotenone powder (Bidepharm, China) was dissolved in commercial sunflower oil. Pure MET powder was obtained from Baoji Guokang Bio-Technology Co., Ltd., China, and dissolved in distilled water. L-DOPA was purchased from Xian Sonwu Biotech Co., Ltd., China, and carbidopa was obtained from Sandoo Pharmaceuticals and Chemicals, China.

Study Approval

All experimental procedures involving animals were conducted by institutional guidelines and were approved by the Ethics Committee of the College of Pharmacy, Mustansiriyah University (Reference No. 179, dated 26 February 2025).

Animals

Thirty albino male mice, weighing between 20 and 30 grams, were acquired from the University of Al-Farahidi's College of Pharmacy, Baghdad, Iraq. The mice were divided into six groups and housed in cages with woodchip bedding in a well-ventilated location, maintained under standard conditions: normal humidity, a 12-hour light/dark cycle, and free access to water and food (commercial pellets).

Drug Administration

To calculate the dosage of rotenone, MET, and L-DOPA/carbidopa, the average body weight of each group was determined. To create a working solution of concentration (1 mg/5 ml), 10 mg of rotenone powder was dissolved in 5 ml of 100% ethanol in a glass tube and carefully agitated with a magnetic stirrer until completely dissolved to determine the proper dosage for administration [12]. MET was used at a dose of 500 mg/kg, dissolved in distilled water, and administered orally via gavage for 30 consecutive days [13]. L-DOPA (100 mg/kg) and carbidopa (25 mg/kg) were dissolved in distilled water for administration [14].

Study Design

Each of the six experimental groups included five mice, which were randomly assigned as follows:

Group 1 (Control group): Nine injections of the vehicle (sunflower oil, 4 mL/kg) were administered subcutaneously to the mice every 48 hours.

Group 2 (Induction group): Received nine doses of rotenone (1 mg/kg, administered every other day subcutaneously) dissolved in 99% pure ethanol.

Group 3 (MET pre-treatment group): Received MET orally every day for three days before rotenone delivery. They then kept receiving MET for seventeen days concurrently with rotenone injections.

Group 4 (MET treatment group): Received nine doses of rotenone (1 mg/kg, every other day, subcutaneously) on day 0 and were subsequently given 500 mg/kg of MET, dissolved in distilled water, orally every day for 30 days following 17 days of rotenone doses.

Group 5 (L-DOPA/carbidopa treatment group): Received nine doses of rotenone (1 mg/kg, every other day, subcutaneously), followed by daily oral administration of L-Dopa (100 mg/kg) with carbidopa (25 mg/kg) diluted in distilled water for 30 days. Group 6 (MET/L-DOPA/carbidopa treatment group): Received nine doses of rotenone (1 mg/kg, every other day, subcutaneously) followed by MET 500 mg/kg + L-Dopa (100 mg/kg). Carbidopa (25 mg/kg) was dissolved in distilled water and administered orally every day for 30 days.

Samples Collection

Following seventeen days of rotenone dosages, the substantia nigra (SN) of control, induction, and pretreatment mice was dissected. Blood samples were collected from each group and kept at –20°C until analysis. The mice were anesthetized via intramuscular injection of ketamine (35 mg/kg) and xylazine (7 mg/kg) to induce deep anesthesia before dissection. For euthanasia, mice were sacrificed by cervical dislocation under deep anesthesia. SN was then dissected and divided for real-time polymerase chain reaction (RT-PCR) analysis, and stored at –40°C in Eppendorf tubes pre-filled with 300 μl of Triazole solution. After receiving MET doses for thirty days, mice in the treatment, standard treatment, and combination groups were euthanized, and blood samples were collected from all mice to measure and compare specific biochemical markers. The serum levels of GSK3β, MDA, and SOD were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Table 1).

RT-PCR measurement of TLR4

The data were standardized to β-actin, the endogenous control or housekeeping gene employed in this investigation. The process was carried out by the guidelines included in the kits. In summary, the TransZol Up Plus RNA Kit (purchased from TransGen Biotech, China) was used to isolate total ribonucleic acid (RNA) from brain tissues. Following that, the

Table 1. Kits and primers used in the study.

Subject	Company	Country	Use
Toll-like receptor 4 (TLR4) primer	TransGen Biotech	China	RT-PCR analysis of TLR4 gene expression
β-actin primer	TransGen Biotech	China	RT-PCR internal control
Glycogen Synthase Kinase 3 Beta (GSK3β) ELISA Kit	Cloud-Clone Corporation	USA	Quantification of GSK3β protein levels in serum
Malondialdehyde (MDA) ELISA Kit	Cloud-Clone Corporation	USA	Measurement of lipid peroxidation (oxidative stress marker)
Superoxide Dismutase (SOD) ELISA Kit	Cloud-Clone Corporation	USA	Assessment of antioxidant enzyme activity

EasyScript® OneStep gDNA Removal and cDNA Synthesis SuperMix Kit (purchased from TransGen Biotech, China) was used to perform genomic deoxyribonucleic acid (gDNA) removal and complementary DNA (cDNA) synthesis.

Quantitative real-time PCR (RT-qPCR) was carried out using a Rotor-Gene Q system (Qiagen, Germany). The thermal cycling conditions were as follows: Initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 30 seconds. A melting curve analysis was conducted at the end of the run to verify the specificity of the amplified products. Expression of TLR4 was quantified using β-actin as the internal reference gene. All reactions were performed in triplicate. The threshold cycle (Ct) values were recorded for each gene, and relative gene expression was calculated using the Efficiency Correction method "Efficiency correction is required for accurate quantitative PCR analysis and reporting" [15]. The following formula was used to calculate the fold change in gene expression: Fold $= 2 - \Delta CT$. Where $\Delta CT = Ct$ of the target gene – Ct of the housekeeping gene.

The primer sequences used for RT-qPCR are listed in Table 2.

Statistical Analysis

All data were analyzed and presented as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test in the statistical package for the social sciences (SPSS) software, version 25.0 (IBM Corp., Armonk, NY, USA). A P-value less than 0.05 was considered statistically significant.

Table 2. Primer sequences used for quantitative real-time polymerase chain reaction analysis of Toll-like Receptor 4 and β-actin genes (5'→3' direction).

Primer	Sequence 5'→3' direction
	β-actin
Forward	CCGCGGGAGACAAGCTT
Reverse	GGAATGGAAGAAGGGCTTGATC
	TLR4
Forward	ATGCATGGATCAGAAACTCAGCAA
Reverse	AAACTTCCTGGGGAAAAACTCTGG

RESULTS

TLR4 expression

In the brain tissue of the rotenone-induced PD model, RT-PCR analysis revealed that TLR4 mRNA expression was low in the control group. In contrast, a significant upregulation of TLR4 was observed in the rotenone-injected group (P-value < 0.05). Pre-treatment with MET (Group 3) resulted in a substantial downregulation of TLR4 mRNA expression compared to the rotenone-only group (P-value < 0.05). Similarly, mice treated with MET alone (Group 4) or L-DOPA/Carbidopa (Group 5) also showed significantly lower TLR4 expression levels compared to the induction group (P-value < 0.05). Furthermore, the combination-treated group (Group 6) of MET and L-DOPA/Carbidopa resulted in a further significant reduction in TLR4 mRNA expression in brain tissue (P-value < 0.05) compared to the induction group. These findings are illustrated in Figure 1.

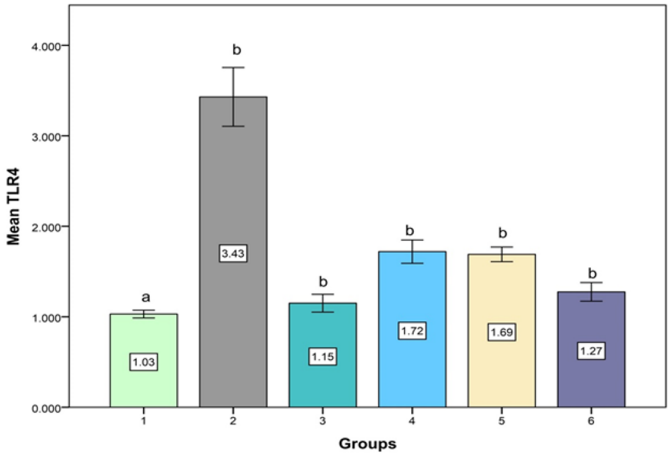

Figure 1. Effect of metformin on toll-like receptor 4 (TLR4) messenger ribonucleic acid expression in male mice with Parkinson's disease. Data are expressed as fold change relative to the control group (Mean ± Standard Deviation, n = 5). Statistical analysis was performed using One-Way Analysis of Variance followed by Tukey's Post Hoc Test. Different lowercase Letters (a, b) above the bars indicate statistically significant differences at P-value < 0.05. Results are presented as bar plots with error bars. Group number: (1) Control, (2) Induction (Rotenone), (3) Pre-treatment (Metformin), (4) Metformin (500 mg/kg), (5) Levodopa/Carbidopa (100/25 mg/kg), (6) Metformin + Levodopa/Carbidopa (500 mg/kg) + (100/25 mg/kg).

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GSK3β

Figure 2 shows that the induction group's mean serum GSK3β level was significantly (P-value < 0.05) higher than that of the control group. Pre-treatment with 500 mg/kg of MET (Group 3) resulted in a significant (P-value < 0.05) reduction in the mean serum GSK3β level relative to the induction group. Additionally, the MET-treated group showed a significant (P-value < 0.05) decrease in serum GSK3β compared to the induction group; however, this effect was non-significantly different (P-value > 0.05) from that observed in the group treated with L-DOPA/carbidopa (100/25 mg/kg, Group 5). The combination-treated group (Group 6) showed the most significant decrease in GSK3β levels (P-value < 0.05), compared to both MET and L-Dopa/Carbidopa monotherapy groups, indicating a potential synergistic interaction between the two treatments.

Oxidative stress biomarkers

MDA levels

The mean serum MDA level in the induction group was significantly higher (P-value < 0.05) than that of the control group. Pre-treatment with 500 mg/kg of MET (Group 3) resulted in a significant reduction (P-value < 0.05) in serum MDA levels compared to the induction group. Furthermore, the mean serum MDA levels in the MET-treated, L-DOPA/carbidopa-treated, and combination-treated groups were significantly lower (P-value < 0.05) than in the induction group. The difference between the MET and L-DOPA/carbidopa groups was not statistically significant (P-value > 0.05) difference (Figure 3).

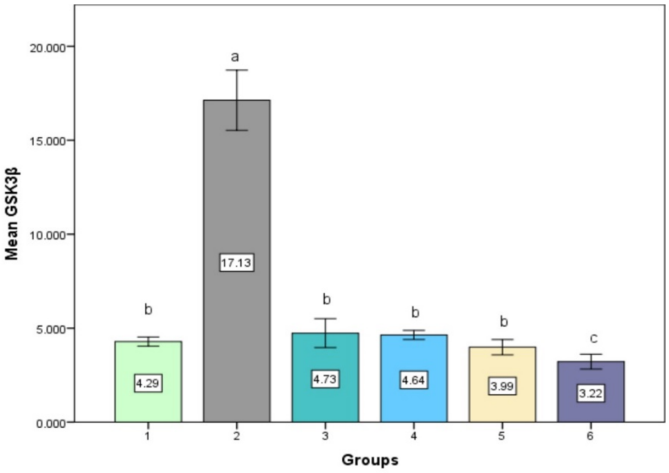

Figure 2. Serum levels of glycogen synthase kinase-3 beta (GSK3β) per ng/mL in male mice with Parkinson's disease treated with metformin. Data are presented as Mean ± Standard Deviation (n = 5). One-way analysis of variance followed by Tukey's Post Hoc Test, was used for statistical analysis. Statistically significant differences are indicated by different lowercase letters (a, b, and c) (P-value < 0.05). Results are displayed as bar charts with error bars. Group number: (1) Control, (2) Induction (Rotenone), (3) Pre-treatment (Metformin), (4) Metformin (500 mg/kg), (5) Levodopa/Carbidopa (100/25 mg/kg), (6) Metformin + Levodopa/Carbidopa (500 mg/kg) + (100/25 mg/kg).

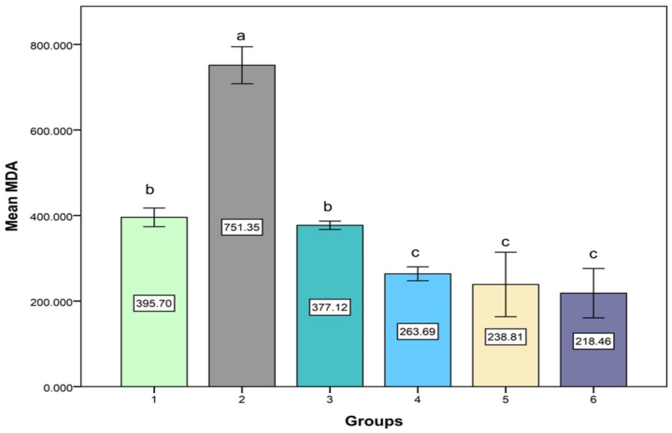

Figure 3. Serum malondialdehyde (MDA) levels (nmol/mL) in male mice with Parkinson's disease treated with metformin. Data are presented as Mean ± Standard Deviation (n = 5). One-Way Analysis of Variance followed by Tukey's Post Hoc test was used for statistical analysis. Significant differences are indicated by different lowercase letters (a, b, and c) at P-value < 0.05. Results are displayed as bar charts with error bars. Group number: (1) Control, (2) Induction (Rotenone), (3) Pre-treatment (Metformin), (4) Metformin (500 mg/kg), (5) Levodopa/Carbidopa (100/25 mg/kg), (6) Metformin + Levodopa/Carbidopa (500 mg/kg) + (100/25 mg/kg).

SOD levels

However, compared to the control group, in the induction group's mean serum SOD level was significantly lower (P-value < 0.05), reflecting oxidative stress induced by rotenone. Pretreatment with MET restored SOD levels, and both MET and L-DOPA/carbidopa significantly increased serum SOD levels compared to the induction group (P-value < 0.05). Notably, the combination group exhibited the highest serum SOD levels among all groups, with a statistically significant increase (P-value < 0.05) compared to the other treatment groups (Figure 4).

DISCUSSION

The present study demonstrated the potential neuroprotective efficacy of MET in a rotenone-induced mouse model of PD. Rotenone, a widely used pesticide, was employed to induce PD-like symptoms due to its ability to inhibit mitochondrial complex I, leading to dopaminergic neuronal degeneration. In the induction group, a significant elevation in GSK3β levels was observed. Elevated GSK3β contributes to oxidative stress and mitochondrial dysfunction in the SN, thereby promoting neurodegeneration and disease progression [16].

Notably, alpha-synuclein (SNCα), a key pathological hallmark of PD, has been shown to modulate GSK3β activity, establishing a self-amplifying feedback loop that exacerbates neurodegenerative processes [17]. Inhibition of GSK3β is known to attenuate tau protein hyperphosphorylation, reduce SNCα accumulation, and prevent neuronal injury [18]. In this context, MET has been reported to exert neuroprotective effects via inhibition of GSK3β, as shown in a recent study where MET reduced active (non-phosphorylated) GSK3β levels in models of neurotoxicity [19]. Our current findings align

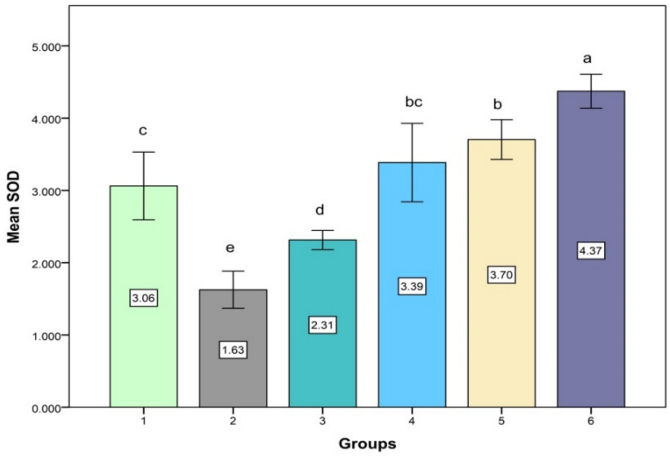

Figure 4. Mean serum levels of superoxide dismutase (SOD) per μg/mL in different experimental groups (n = 5). Data are presented as Mean ± Standard Deviation. Statistical analysis was performed using One-Way Analysis of Variance, followed by Tukey's Post Hoc test. Different lowercase letters (a–e) Above the bars indicate statistically significant differences at P-value < 0.05. Groups sharing at least one common letter (For example 'bc' and 'b and c') are not significantly different from each other. Group number: (1) Control, (2) Induction (Rotenone), (3) Pre-treatment (Metformin), (4) Metformin (500 mg/kg), (5) Levodopa/Carbidopa (100/25 mg/kg), (6) Metformin + Levodopa/Carbidopa (500 mg/kg) + (100/25 mg/kg).

with these reports, showing that MET reduced GSK3β levels, suggesting its role in protecting dopaminergic neurons.

TLR4 is a member of the toll-like receptors family that triggers pro-inflammatory responses to invasive pathogens [20]. TLR4 signaling may increase gut permeability and inflammation, which can cause SNCα aggregation in the gut or brain and contribute to the pathophysiology of PD [21]. Our study showed that rotenone-treated mice exhibited a significant (P-value < 0.05) increase in TLR4 mRNA expression compared to the control group. Our 30-day MET treatment regimen significantly attenuated TLR4 expression. Previous in vitro and in vivo studies have suggested that MET attenuates inflammatory responses by inhibiting TLR4/NF-κB signaling, possibly via AMPK activation. However, direct evidence in PD models has been limited [22]. Similarly, MET has been reported to inhibit the TLR4/TRAF6/NLRP3 inflammasome pathway in non-neuronal models such as diabetic nephropathy [23]. However, to our knowledge, this is the first study to provide direct evidence of MET's ability to downregulate TLR4 expression in a neurodegenerative model of Parkinsonism. This novel finding highlights the translational potential of MET in modulating central inflammatory pathways relevant to the pathogenesis of PD.

Oxidative stress is a key contributor to PD pathology, characterized by reduced SOD activity and elevated MDA levels. Our findings confirm that rotenone exposure led to a significant decline in SOD and an increase in MDA. Reduced SOD causes oxidative damage that results in the death of neurons, while high MDA disrupts membrane structure, increases lipid peroxidation, and impairs the functionality of proteins and DNA [24, 25]. Excessive ROS buildup the formation of toxic protein aggregates, leading to mitochondrial dysfunction and neuronal cell death. These are linked to PD and other neurodegenerative diseases [26]. Interestingly, MET administration counteracted these effects by enhancing the antioxidant defense system, as evidenced by preserved SOD activity and reduced MDA levels, suggesting a protective role against oxidative stress-mediated neuronal damage.

This study has several limitations that should be acknowledged to provide a comprehensive interpretation of the findings. Firstly, the use of a rotenone-induced PD model in mice, while well-established, may not fully replicate the complexity of the human condition, limiting the generalizability of the results. Secondly, the study duration was relatively short (30 days), which may not adequately reflect the long-term effects of MET treatment or the chronic progression of PD. Thirdly, it is advisable to consider using a lower dose of MET (e.g., 250 mg/kg, which is half the dose used in this study) when combined with L-DOPA/carbidopa, to prevent an excessive decrease in certain biomarkers and help maintain values within the normal physiological range. Further studies are needed to confirm the appropriate dosing.

CONCLUSION

MET demonstrated significant antioxidant and anti-inflammatory effects in a rotenone-induced parkinsonism model. It reduced serum MDA and GSK-3β levels, increased SOD activity, and downregulated TLR4 mRNA expression. These results highlight its neuroprotective potential in this experimental setting.

ETHICAL DECLARATIONS

Acknowledgments

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Ethics Approval and Consent to Participate

All experimental procedures involving animals were conducted in accordance with institutional guidelines and were approved by the Ethics Committee of the College of Pharmacy, Mustansiriyah University (Reference No. 179, dated 26 February 2025).

Consent for Publication

Not applicable.

Availability of Data and Material

Data generated during this study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that there is no conflict of interest.

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Authors' Contributions

All authors contributed equally to the conception and design of the study, data collection, analysis, and interpretation of results, manuscript drafting, and final revision. All authors read and approved the final version of the manuscript.

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