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Original Article

Thymoquinone and madecassoside improve motor function in a rotenone-induced mouse model of early Parkinson's disease: Role of dopamine, alpha-synuclein and mBDNF

Shinta Kusumawati[1,2], Agustina T. Endharti[3,4], Farhad Balafif[5], Shahdevi N. Kurniawan[6], Aris Rosidah[7], Rabjhany Anaqah[8], Izzunazel A. Barqillah[9] and Husnul Khotimah[10*]

[1]Doctoral Program in Medical Science, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia; [2]Departement of Neurology, Faculty of Medicine, Universitas Islam Malang, Malang, Indonesia; [3]Department of Parasitology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia; [4]Biomedical Central Laboratory, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia; [5] Department of Neurosurgery, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia; [6]Department of Neurology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia; [7]Departement of Anatomical Pathology, Faculty of Medicine, Universitas Islam Malang, Malang, Indonesia; [8]Master Program in Biomedical Sciences, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia; [9]Faculty of Medicine, Universitas Islam Malang, Malang, Indonesia; [10]Department of Pharmacology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia

*Corresponding author: husnul_farmako.fk@ub.ac.id

Abstract

Parkinson's disease is a progressive, incurable neurodegenerative disorder characterized by the degeneration of dopaminergic neurons and pathological aggregation of α-synuclein in the midbrain, leading to motor dysfunction. Thymoquinone (TQ), an active compound from *Nigella sativa*, has demonstrated antioxidant properties that may reduce dopamine degradation, while madecassoside (MA), a triterpenoid component of *Centella asiatica*, exhibits neuroprotective effects. To date, no study has investigated the combined effects of TQ and MA in a Parkinson's disease model. The aim of this study was to evaluate the synergistic neuroprotective potential of TQ and MA on motor function, dopamine levels, α-synuclein accumulation, and mature brain-derived neurotrophic factor (mBDNF) expression in a rotenone (ROT)-induced mouse model of early Parkinson's disease. Rotenone (2.5 mg/kg BW) was administered subcutaneously for two weeks to induce Parkinson's disease, while TQ alone, MA alone and combination of TQ and MA at various doses, as well as a reference drug (pramipexole) were given every 48 hours concurrently with rotenone. Motor symptoms were assessed through behavioral tests, including the open field test (OFT), beam walking test, and hanging wire test; midbrain dopamine levels were quantified via enzyme-linked immunosorbent assay (ELISA), α-synuclein expression was assessed using Western blotting, and immunohistochemistry was used to detect mBDNF-positive cells in the cerebral cortex. The combination of TQ and MA significantly increased midbrain dopamine levels and improved locomotor activity, as shown by increased total distance traveled and mean velocity in ROT-induced mice. Biochemically, this combined treatment reduced α-synuclein expression, suggesting attenuation of early pathological aggregation typically observed in Parkinson's disease. Although the increase in mBDNF expression in the cerebral cortex was not statistically significant, it was higher in the TQ-MA treatment group compared to controls and other groups. Collectively, these results highlight the therapeutic potential of TQ and MA in combination to counteract both motor deficits and early neurochemical disruptions in a ROT-induced model of Parkinson's disease.

Keywords: Parkinson, dopamine, α-synuclein, motoric, thymoquinone

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Introduction

Parkinson's disease is a neurologic condition caused by chronic inflammation and oxidative stress, resulting in α-synuclein aggregation and dopaminergic cell death [1,2]. The pathological characteristic of Parkinson's disease is the degeneration of dopaminergic neurons in the striatum nigra caused by the accumulation and spread of Lewy bodies and nerve sheath inclusions due to misfolding of fibers derived from a neurotoxic form of α-synuclein [3]. The α-synuclein inhibits neurotrophic activity of mature brain-derived neurotrophic factor (mBDNF) in the substantia nigra by downregulating mBDNF expression and competitively inhibiting mBDNF signaling at the receptor level [4]. In both the peripheral and central nervous systems, mBDNF is essential for the survival, differentiation, and proliferation of neurons [5]. The neurotrophin family member mBDNF has a high affinity for the tropomyosin-related kinase B (TrkB) receptor, which enhances spine complexity, enables long-term potentiation, and supports cell survival [6]. mBDNF is a mature isoform of BDNF produced from the cleavage of pro-BDNF that has been shown to promote the growth and survival of dopamine neuron synapses in the substantia nigra [4,7]. Dopaminergic neurons store and release dopamine, a neurotransmitter that regulates behavior, emotion, and cognitive functioning. Dopamine is a monoamine neurotransmitter with key regulatory functions in the central nervous system [8,9]. The onset of Parkinson's disease cellular neuropathology can occur decades before the onset of motor symptoms. Loss of dopaminergic neurons occurs in approximately 30% of cases by the time the first symptoms of Parkinson's disease appear [10]. Dopamine is more evenly distributed in the olfactory bulb, midbrain substantia nigra, hypothalamus, ventral tegmental area, retina, and periaqueductal gray regions than other monoamines [11,12]. Mechanisms involved in the pathogenesis of sporadic Parkinson's disease include neuroinflammation and oxidative stress, dysfunction of the innate and adaptive immune systems, impaired mitochondrial activity, genetic mutations, denaturation and aggregation of intracellular proteins and environmental factors [13].

Based on epidemiological studies [14], exposure to pesticides, especially rotenone (ROT) and paraquat, increases the risk of Parkinson's disease. Pesticide exposure causes a 95% risk of sporadic Parkinson's disease [15]. ROT has an advantage over other pesticides for inducing Parkinson's disease experimentally because it can develop the main pathogenic characteristics of clinical Parkinson's disease [16,17]. ROT easily penetrates biological membranes, including the blood-brain barrier, due to its strong hydrophobicity [18]. ROT has been found to selectively degenerate dopaminergic neurons and terminals in the substantia nigra and striatum, leading to motor symptoms [18,19]. Low doses of ROT can alter calcium signaling, and induce oxidative stress, apoptosis, and α-synuclein aggregation that are characteristic of the early stages of Parkinson's disease [20].

Currently, Parkinson's disease remains an incurable disease, and Parkinson's treatment management is needed to slow and stop the progression of the disease [13]. Conventional treatment cannot inhibit the progression of Parkinson's disease; furthermore, it often causes side effects and motor complications such as dyskinesia, choreoathetosis, and fluctuations in motor function [13,21]. Therefore, more effective and safer alternative treatments are highly expected. The primary goal of Parkinson's disease treatment is to develop therapeutic strategies to prevent neurodegeneration. Natural products' anti-inflammatory and anti-oxidant properties have sparked growing interest in their usage for the management of neurodegenerative illnesses [22,23]. Thymoquinone (TQ) is an active substance found in black cumin oil (*Nigella sativa*), accounting for approximately 30% to 48% of the total [24,25]. TQ exhibits anti-inflammatory, antioxidant, immunostimulatory, and anticancer effects [26,27]. TQ is lipophilic, which allows it to pass through the blood-brain barrier, and therefore, it has the potential to target the central nervous system [28]. Madecassoside (MA) is the primary bioactive saponin found in *Centella asiatica* [20,29]. *C. asiatica*, also known as *pegagan*, is a tropical plant belonging to the Apiaceae family found in tropical and subtropical climates throughout Asia, including Malaysia, India, and China [30]. *C. asiatica* has also been linked to neurological advantages such as neuroprotection, memory enhancement, antidepressant activity, and anxiolytic effects [26]. There has been no previous study examining the combination of TQ and MA in Parkinson's disease. Therefore, the aim of this study was to explore the synergistic neuroprotective effects of TQ and MA on

dopamine levels, α-synuclein aggregation, mBDNF and motoric function in a ROT-induced mouse model of Parkinson's disease.

Methods

Study design and setting

A post-test-only experimental study was conducted at the Animal House Integrated Pharmacology Laboratory and the Biomedical Laboratory Facility, Medical Faculty, Universitas Brawijaya, Malang, Indonesia, in 2024 using a total of 24 male Swiss mice (*Mus musculus*). The inclusion criteria were male Swiss mice, healthy and actively moving, while the exclusion criteria were aggressive mice that attacked other mice, unable to survive until the experiment was completed, and mice that were declared sick. The Parkinson's model was created by inducing the animals with neurotoxic ROT at a dose of 2.5 mg/kg every 48 hours given for two weeks [16]. The treatments with TQ, MA and pramipexole (PRX, positive control) were given concurrently with ROT for two weeks. A detailed scheme of the study design is presented in (**Figure 1)**.

Body weight of each animal was monitored twice, before and after therapeutic phase. Behavioral and motor assessments were performed the day following the therapeutic period ended. The mice were then anesthetized with a single dose of ketamine (40–50mg/kg BW) by intramuscular injection into the muscles of the hind or forelimbs, and the brains of sacrificed mice were extracted and divided into two components. One component (midbrain) was dissolved in phosphate buffer (pH 7.5) and utilized for biochemical analysis, while the other component was used for histopathological and immunohistochemical examinations on samples kept in 10% buffered formalin. Biochemical markers, dopamine levels, α-synuclein and mBDNF expressions were then measured (**Figure 1**).

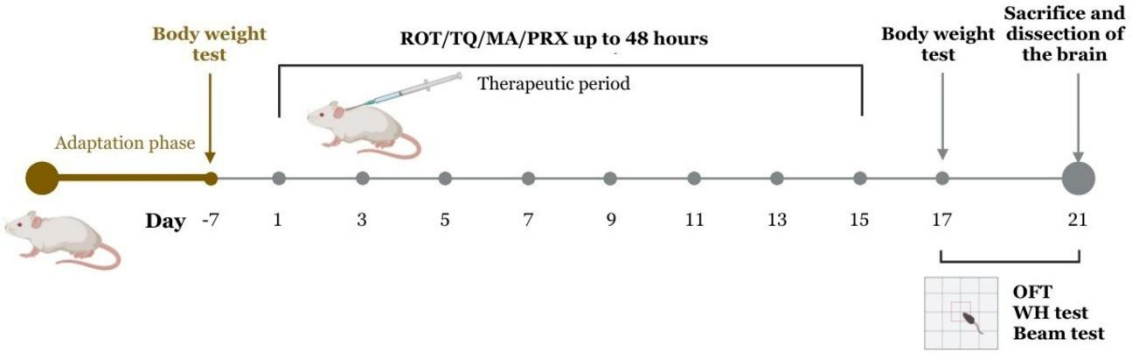

Figure 1. Scheme displaying the study design. MA: madecassoside; OFT: open field test; PRX: pramipexole; ROT: rotenone; TQ: thymoquinone; WH: wire hanging.

Animals and husbandry

Male Swiss mice (*Mus musculus*), aged 8–12 weeks and weighing 25–30 g, were used in this study (n=24). The animals were obtained from the Department of Animal Husbandry and Fisheries of Karanganyar Regency, in collaboration with the Bioscience Laboratory at Universitas Brawijaya, Malang, Indonesia. Animal procurement complied with Certificate of Cultivation No. 524/082.19/I/2019, with strain identification code K-0003. The original certificate holder was listed on the kemuning.co.id website. The mice were housed in cages at 25–30°C, provided with unlimited access to water, and fed a standard diet.

Parkinson's disease induction

The mice were used as Parkinson's model animals after being induced with neurotoxic ROT at a dose of 2.5 mg/kg every 48 hours dissolved in dimethyl sulfoxide (DMSO) and injected subcutaneously (SC) in the nape area of the mice, given for two weeks [16]. To determine the decrease in Parkinson's motor symptoms in ROT-induced mice, locomotor activity was assessed using the open field test (OFT), motor coordination using the beam running test, and muscle strength using the hanging wire test.

Study groups and treatments

The animals were divided into eight groups, with three mice in each group. The details of study groups and the treatments received for each group are presented in (**Figure 2**). The study groups and treatments were as follows: (1) healthy control group, which received no treatment; (2) ROT group, in which mice were administered rotenone (ROT, 2.5 mg/kg body weight, SC) every 48 hours for two weeks to induce Parkinson's disease[1]; (3) RT15 group, in which mice were treated with ROT (2.5 mg/kg, SC) and TQ (15 mg/kg, orally) every 48 hours for two weeks[2]; (4) RMA group, in which mice received ROT (2.5 mg/kg, SC) and MA (15 mg/kg, orally) every 48 hours for two weeks[3]; (5) RT7.5MA group, in which mice were treated with ROT (2.5 mg/kg, SC), TQ (7.5 mg/kg, orally), and MA (15 mg/kg, orally) every 48 hours for two weeks; (6) RT10MA group, in which mice received ROT (2.5 mg/kg, SC), TQ (10 mg/kg, orally), and MA (15 mg/kg, orally) every 48 hours for two weeks; (7) RT15MA group, in which mice were administered ROT (2.5 mg/kg, SC), TQ (15 mg/kg, orally), and MA (15 mg/kg, orally) every 48 hours for two weeks; and (8) RPRX group, in which mice were treated with ROT (2.5 mg/kg, SC) and pramipexole (PRX, 1 mg/kg, orally) every 48 hours for two weeks (**Figure 2**). PRX (MedChemExpress, Monmouth Junction, NJ, USA) was administered to the RPRX group at a dose of 1 mg/kg body weight every 48 hours via oral gavage. TQ (MedChemExpress, Monmouth Junction, NJ, USA) was prepared in polyethylene glycol 200 (Sigma-Aldrich, St. Louis, MO, USA) and given every 48 hours for two weeks by oral gavage at designated doses based on the study group. Madecassoside (MedChemExpress, Monmouth Junction, NJ, USA) was administered at a dose of 15 mg/kg every 48 hours to the RMA group and all TQ-MA combination groups.

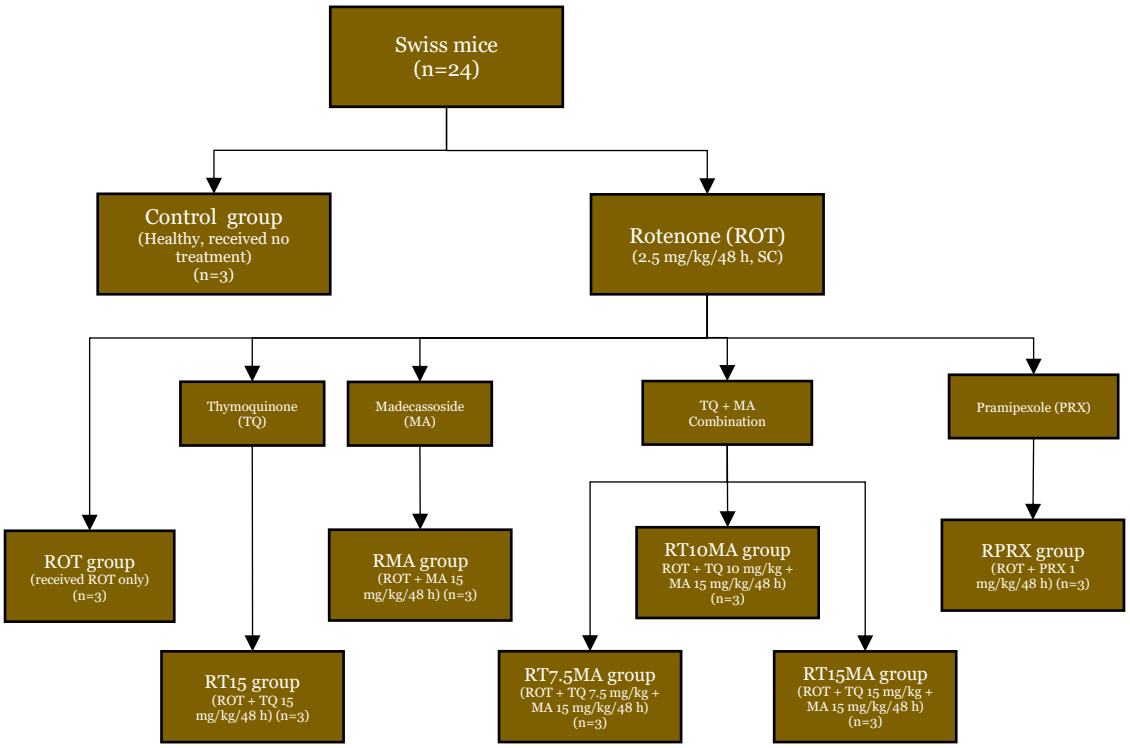

Figure 2. Diagram showing study groups and the treatment of each group.

Study outcomes

The primary outcome of this study was the evaluation of changes in ROT-induced Parkinsonian motor symptoms in Swiss mice, as assessed through behavioral tests, including OFT, beam walking test, and hanging wire test. Biochemical markers characteristic of the Parkinson's model were also examined, including dopamine levels and α-synuclein expression in the midbrain, as well as mBDNF expression in the cortex.

Behavior assessments

Open field test (OFT)

To evaluate the effects of ROT exposure on locomotor activity, total distance traveled, latency to first movement (immobility time), and time spent in the central zone were assessed using OFT. This test also served to evaluate voluntary movement and anxiety-like behavior [31]. The experiment was performed in a square arena divided into 16 equal sections (**Figure 3**). Each mouse was placed in the center of the arena and allowed to explore for three minutes before the next subject was introduced. The arena was thoroughly cleaned between trials to eliminate residual odors that might influence behavior [16]. A video camera continuously recorded the animals' movements over a 360-second test period [31]. To minimize interference, the experimenter remained outside the animals' field of view [23,32]. Locomotor activity was analyzed using EthoVision XT software (Noldus Information Technology, Wageningen, Netherlands). Contralateral rotations were manually recorded by two independent observers who were blinded to the experimental conditions.

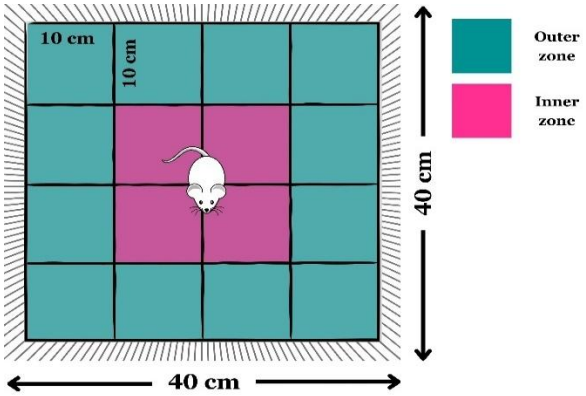

Figure 3. Schematic representation of the open field test (OFT). The OFT arena consists of a square container divided into 16 equal sections, comprising an outer zone and an inner zone.

Beam walking test (Beam test)

The beam-walking test was employed to assess motor coordination and balance. Each mouse was required to traverse a 50 cm wooden beam suspended between a designated starting point and its home cage (**Figure 4**). Mice were placed at the starting point, and the time taken to reach the goal (up to 60 seconds) was recorded, along with the number of foot slips—defined as any instance in which a paw failed to maintain contact with the upper surface of the beam [1,23]. The number of slips recorded during the beam test was further evaluated using a seven-category rating score (0–6) as follows: 0 – the mouse failed to remain on the beam; 1 – remained on the beam without moving; 2 – attempted to cross but fell; 3 – crossed with 4–6 slips; 4 – crossed with 2–3 slips; 5 – crossed with a single hindlimb slip; and 6 – crossed without any hindlimb slips [23].

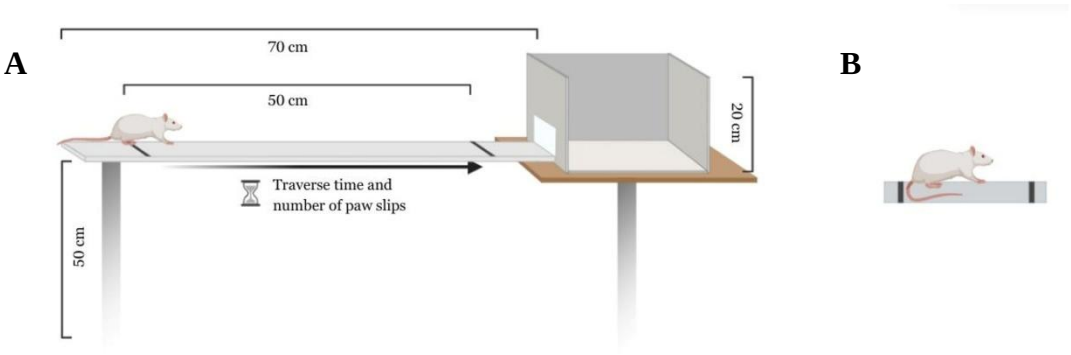

Figure 4. Schematic representation of the beam-walking test to evaluate motor coordination. The test consisted of two components: (A) measuring travel time (seconds) and (B) counting the number of slips recorded during the test [1,33].

Wire hanging test

A wire mesh grip test was used to assess muscle strength. Before testing, each mouse was acclimated to the wire mesh. The mesh was then inverted, and the longest hanging time before the mouse fell into the cage below was recorded. Scoring was based on hanging duration as follows: 1 – falling between 1–10 seconds; 2 – falling between 11–25 seconds; 3 – falling between 26–60 seconds; and 4 – falling after 60 seconds [16,34].

Dopamine level measurement by enzyme-linked immunosorbent assay (ELISA) method

Dopamine levels of the midbrain were measured using an enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's instructions (Elabscience, Houston, TX, USA). All procedures adhered to the provided protocol. Briefly, diluted specimens (1:2) were added in triplicate to microtiter plate wells coated with a dopamine-specific antibody and incubated at 37°C for two hours. After washing to remove unbound substances, a biotin-conjugated antibody was added, followed by incubation at 37°C for 45 minutes. The wells were then treated with avidin-conjugated horseradish peroxidase (HRP) for 30 minutes at 37°C, washed, and a stop solution was applied to terminate the reaction. Absorbance was measured at 450 nm using a Zenix-320 microplate reader (Sumifin Citra Abadi, Tangerang, Indonesia), and dopamine levels were expressed as pg/mg protein [35].

Western blot analysis of alpha-synuclein

Midbrain tissues were homogenized and lysed utilizing PRO-PREPTM Extraction of Protein Solution (Intron Biotechnology Inc., Seongnam, Republic of Korea), which contained phenylmethylsulfonyl fluoride (PMSF). The lysates were centrifuged at 11,000 × g for 20 min at 4°C, and the supernatant was transferred to a 1.5 mL microtube, separated by SDS-PAGE-actin on a 12% gel, SDS-PAGE-alpha-synuclein on a 10% gel for 100 min (Bio-Rad Laboratories, California, USA) and then transferred by semidry transfer (Bio-Rad Laboratories, California, USA) for 20 v 120 min. The following primary antibodies were used: α-synuclein Ser129 polyclonal antibody (Bioss Inc, Woburn, MA, USA). The membranes were incubated with goat anti-rabbit IgG (H+L) secondary antibody, biotin (Thermo Fisher Scientific, Waltham, MA, USA) at 1:200,000 dilution. Primer antibody beta-actin (1:5,000; Santa Cruz Biotechnology, Dallas, Texas, USA) was used as an internal loading control. The membranes were incubated using anti-mouse IgG horseradish peroxidase conjugate (Rockland Immunochemicals Inc, Pottstown, PA, USA) secondary antibodies for one hour at room temperature. Western blot images were captured using a chemiluminescence ImageQuant LAS 500 (GE Healthcare, Chicago, IL, USA) [36].

Immunohistochemistry analysis of mature BDNF

The distribution of mBDNF cells in brain tissue was assessed using the mBDNF Primary Antibody Kit (Thermo Fisher Scientific, Waltham, MA, USA). Mouse cerebral cortex sections were transferred to 20% sucrose in 0.1 M PBS, allowed to sink, frozen, and sectioned at 30 µm using a freezing microtome (Leica, Wetzlar, Germany). Free-floating sections were washed in 0.1 M PBS (3×5 min), incubated in 0.2% hydrogen peroxide for 20 min to block endogenous peroxidase, and immersed in PBS at 37°C for 30 min. Sections were then incubated at 4°C for 48 h with a polyclonal neurotrophin antisera to boiling mBDNF (1:1000), followed by incubation with a biotin-conjugated secondary antibody for one hour at room temperature. After washing (3×5 min in 0.1 M PBS), sections were treated with streptavidin-HRP for 40 min, and immunoreaction products were visualized using a staining solution (0.04% 3,3-diaminobenzidine, 0.06% nickel sulfate, 0.06% hydrogen peroxide) for 10 min. Sections were mounted, dehydrated, coverslipped, and observed under a light microscope (Olympus, Tokyo, Japan) at 400× magnification [37]. mBDNF expression was assessed in five fields per slide and classified as negative (no cells), weakly positive (≤25%), moderately positive (26–50%), or strongly positive (>50%) [38].

Statistical analysis

Data were analyzed and presented as mean±standard deviation (SD) using RStudio (Posit PBC, Boston, MA, USA) and ImageJ (National Institutes of Health, Bethesda, MD, USA). One-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests, was employed to

assess differences among and between groups, respectively, with statistical significance set at $p<0.05$. The ANOVA test was applied to analyze the OFT, beam test, and wire hanging test, with significance set at $p<0.05$.

Results

Effect of thymoquinone and madecassoside on body weight in ROT-induced mice

To validate the non-specific effects of ROT on body weight, we assessed mice weight before and after treatment. Body weight increased in the control (31.3±4.7 vs 35.2±3.6, $p=0.025$), ROT (26.6±1.8 vs 33.8±4.3, $p=0.038$), RT15 (29.8±2.8 vs 31.9±1.9, $p=0.05$), RMA (28.9±0.4 vs 33.3±2.6, $p=0.116$), and RT10MA (30.6±1.9 vs 33.6±0.2, $p=0.331$) groups, while it decreased in the RT7.5MA (31.7±3.1 vs 31.2±2.9, $p=0.1$), RT15MA (30.3±3.5 vs 29.8±5.2, $p=0.814$), and RPRX (34.1±0.8 vs 30.8±1.8, $p=0.151$) groups. However, there was no significant difference in body weight among groups before and after treatment ($p>0.05$).

Effect of thymoquinone and madecassoside on motoric function in ROT-induced mice

In this study, motor function was assessed using OFT which consists of total distance, mean velocity, frequency inner zone and latency to first zone variables. For each of these variables, normality test was performed using Shapiro-Wilk test and homogeneity test using Levene's Test. A p-value of >0.05 was obtained for each test, which means the data was normally distributed and homogeneous. To analyze the differences among the study groups, each OFT variable was tested using ANOVA test. The results showed significant differences among the groups for total distance ($p=0.007$) and mean velocity ($p=0.005$) (**Table 1**). In contrast, frequency of the inner zone ($p=0.104$) and latency to the first inner zone ($p=0.524$) showed no significant differences (**Table 1**). A post-hoc Dunn's test was performed to identify specific group differences.

Table 1. Effect of thymoquinone (TQ) and madecassoside (MA) on motor and exploratory behaviors using open field test (OFT) examination

Variable OFT	Study group Mean±SD								p-value [a]
	Control	ROT	RT15	RMA	RT7.5MA	RT10MA	RT15MA	RPRX	
Total distance (mm)	1894.4±670.2	725.9±216.5	1686.99±570.6	1128.3±85.8	1145.3±193.9	1111.7±0.1	1063.2±225.3	758.2±107.2	0.007*
Mean velocity (mm/s)	6.46±2.1	2.53±0.6	5.7±1.9	3.74±0.2	3.85±0.6	3.7±0.17	3.5±0.7	2.6±0.2	0.005*
Frequency inner zone (n)	30±13	4.3±3.5	16.5±10.5	9.5±1.5	14.3±5.7	8±0	10.3±9.5	7.5±0.5	0.104
Latency to first inner zone (s)	29.0±1.8	82.7±85.9	42.7±38.8	20.79±12.4	14.57±5.7	27.2±20.9	69.3±77.5	34.9±8.3	0.541

Control group: received no treatment; ROT group: received ROT only (2.5 mg/BW); RT15 group: received ROT (2.5mg/BW) and TQ (15 mg/BW); RMA group: received ROT (2.5 mg/BW) and MA (15 mg/BW); R7.5MA group: received ROT (2.5 mg/BW),TQ (7.5mg/BW) and MA (15mg/BW); RT10MA group: received ROT (2.5 mg/BW), TQ (10 mg/BW), and MA (15 mg/BW); RT15MA group: received ROT (2.5 mg/BW), TQ (15 mg/BW), and MA (15 mg/BW); RPRX group: received ROT (2.5 mg/BW) and pramipexole (PRX) (1 mg/BW).

[a] Analyzed using Anova test.

*Statistically significant at $p<0.05$.

The OFT results revealed the treatments' impact on motor and exploratory behaviors. The control group was significantly more active than the other groups in terms of total distance traveled ($p<0.05$). By contrast, the ROT group exhibited the shortest distance, reflecting severe locomotor impairment. Among the treatments, RT15 showed the most significant recovery compared to the ROT group ($p<0.05$). However, RMA, R7.5MA, RT10MA, RT15MA and RPRX showed a slight recovery but were not significant compared to the ROT group (**Figure 5A**). The control group had a significantly greater mean velocity than the other groups ($p<0.05$), while the

ROT group had the lowest value. RT15 and RPRX had a significant improvement in velocity compared to the ROT group (p<0.05) (**Figure 5B**).

Exploratory behavior, measured by frequency in the inner zone and latency to the first inner zone, showed no significant differences between groups (p>0.05) (**Figure 5C, 5D**). The control group exhibited higher exploratory activity for frequency in the inner zone compared to all other groups, with modest trends toward improvement observed in the RT10MA and RT15MA groups. For latency to the first inner zone, the ROT group showed prolonged latency compared to the control group, while the RT10MA group showed a slight reduction, suggesting partial recovery. These results highlight that RT15 provided the most consistent improvement in motor function, particularly in total distance and velocity, although it did not fully restore activity to control levels. Other treatments, including RPRX, showed limited efficacy in restoring motor and exploratory behavior.

The analysis of tracking locomotor activity also indicated that the ROT group experienced a decreased locomotor activity compared to the control group and other treatment groups (**Figure 6A**). RT15 showed more visual tracks of locomotor activity compared to the ROT group and other groups, while the combination group of TQ and MA at RT7.5MA, RT10MA and RT15MA showed similar visual locomotor track patterns (**Figure 6E-G**). The combination groups of TQ and MA at doses TQ7.5 MA 15 and TQ10 MA15 appeared to increase locomotor activity compared to ROT group only. Even though these trends were not statistically significant, they suggested potential effects of moderate-dose combinations that warrant further investigation.

The results of the present study for the beam test, analyzed by one-way ANOVA, showed no significant differences were observed between the groups (p>0.05), although trends were noted. The RMA group and RT7.5MA group showed a longer transversal time, indicating impaired motor coordination, whereas the RT15, RT10MA and RPRX groups showed a shorter transversal time, indicating slight improvements (**Figure 7A**). The score for slips off the beam, analyzed using the Kruskal-Wallis test, revealed no significant differences among the groups (p>0.05), indicating that all treatments had comparable performance in minimizing foot slips (**Figure 7B**). In the wire hanging test, no significant differences were observed between groups (p>0.05), which assesses muscle strength. However, a descriptive analysis showed better performance in the RPRX group, although the variations were not statistically significant. These findings suggested that while some treatments, particularly RT10MA and RPRX, showed trends toward improved motor coordination and muscle strength, further studies are needed to confirm these effects.

Effect of thymoquinone and madecassoside on alpha-synuclein expression in midbrain

Western blot analysis revealed that α-synuclein expression in the 100 kDa band was significantly reduced in the control group compared to the ROT group (0.13±0.06 vs. 0.53±0.08; p=0.000). Similarly, significantly reduced expression levels were observed in the RT15 (0.24±0.03), RT10MA (0.28±0.01), compared to the ROT group (all p<0.05) (**Figure 9A and 9C**). The RMA (0.49±0.01), RT7.5MA (0.43±0.04), RT15MA (0.46±0.06) and RPRX (0.36±0.15) also showed reduced α-synuclein expression, but not significantly (p>0.05) (**Figure 9C**). All groups showed equivalent actin expression at 12%, which was used as a loading control (**Figure 9B**). One-way ANOVA indicated that the differences in mean α-synuclein expression were statistically significant (p=0.000) (**Figure 9C**). While co-administration of TQ and MA appeared to significantly reduce α-synuclein aggregation on middle dose (TQ 10mg/BW and MA 15mg/BW) in ROT-induced mice, the lower dose and higher dosed were not significant and warrant further investigation.

Effect of thymoquinone and madecassoside on dopamine level in midbrain

Dopamine levels varied significantly across experimental groups (p<0.05, one-way ANOVA) (**Figure 8**). The control group exhibited significantly higher dopamine levels compared to all other groups, whereas the ROT group showed the lowest level, indicating that ROT induced a substantial depletion of dopamine. Treatment with RT15MA resulted in a significant improvement in dopamine concentration compared to the ROT group (125.09± 2.05 vs. 43.19± 16.94 pg/mg, p=0.0004), although levels remained below those observed in the control group. Similarly, treatment with RT10MA also significantly increased dopamine levels relative to

the ROT group (121.56± 8.38 vs. 43.19± 16.94 pg/mg, p=0.0005). The RPRX group, which received the reference drug, demonstrated the greatest improvement (154.44± 27.34 vs. 43.19± 16.94 pg/mg, p=0.0001), although dopamine levels did not fully return to control values. These findings suggested that while RPRX exhibited the strongest effect in restoring dopamine levels, co-administration of TQ and MA, particularly at higher doses, also resulted in a significant recovery of dopamine levels.

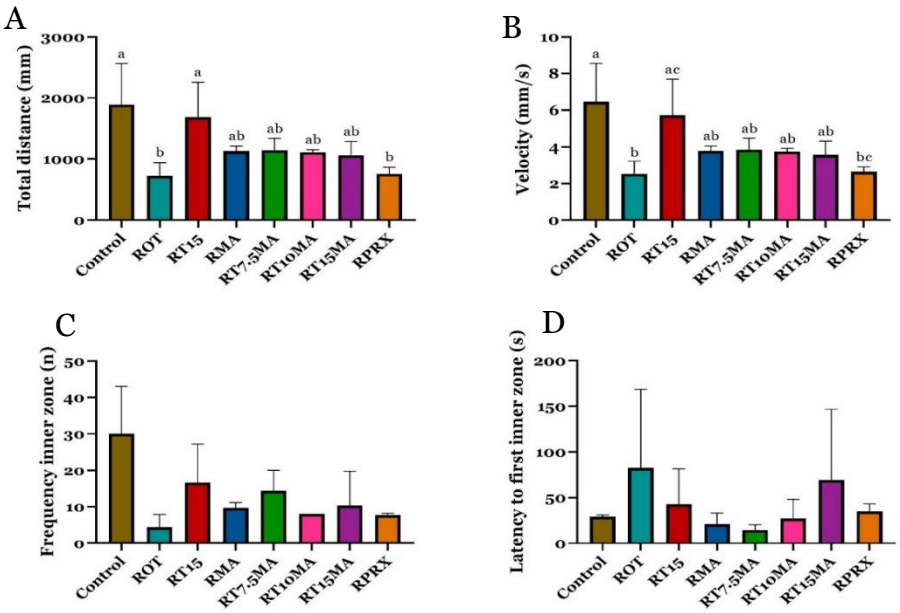

Figure 5. Effect of thymoquinone (TQ) and madecassoside (MA) on motoric function using open field motor and exploratory behaviors in rotenone (ROT)-induced mice: (A) total distance, (B) velocity, (C) frequency inner zone, and (D) latency to the first inner zone. Results are expressed as the mean±SD. Data were analyzed using the Kruskal-Wallis test and significant differences between groups were indicated by different letters on the top of the bars (p<0.05). Control group: received no treatment; ROT group: received ROT only (2.5 mg/BW); RT15 group: received ROT (2.5mg/BW) and TQ (15 mg/BW); RMA group: received ROT (2.5 mg/BW) and MA (15 mg/BW); R7.5MA group: received ROT (2.5 mg/BW), TQ (7.5mg/BW) and MA (15mg/BW); RT10MA group: received ROT (2.5 mg/BW), TQ (10 mg/BW), and MA (15 mg/BW); RT15MA group: received ROT (2.5 mg/BW), TQ (15 mg/BW), and MA (15 mg/BW); RPRX group: received ROT (2.5 mg/BW) and pramipexole (PRX) (1 mg/BW).

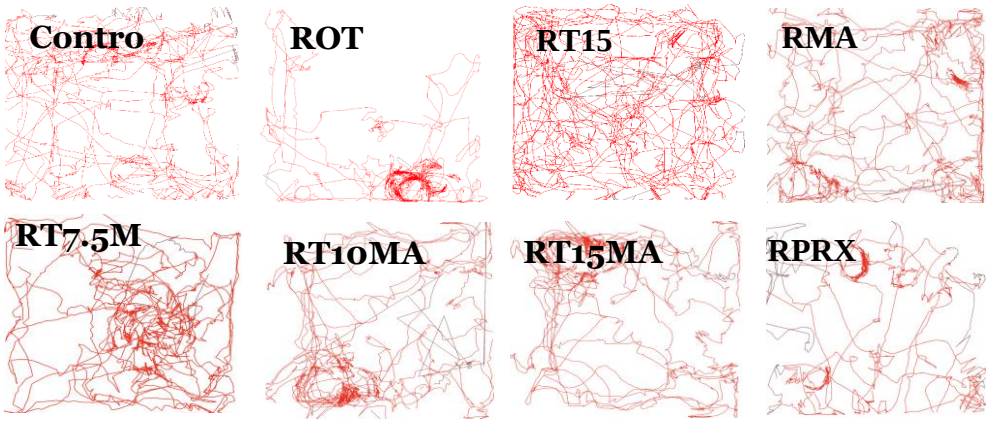

Figure 6. Representative of tracking plot of locomotor activity of mice treated with thymoquinone (TQ) and madecassoside (MA) among rotenone (ROT)-induced mice in open field test (OFT). Control group: received no treatment; ROT group: received ROT only (2.5 mg/BW); RT15 group: received ROT (2.5mg/BW) and TQ (15 mg/BW); RMA group: received ROT (2.5 mg/BW) and MA (15 mg/BW); R7.5MA group: received ROT (2.5 mg/BW),TQ (7.5mg/BW) and MA (15mg/BW); RT10MA group: received ROT (2.5 mg/BW), TQ (10 mg/BW), and MA (15 mg/BW); RT15MA group: received ROT (2.5 mg/BW), TQ (15 mg/BW), and MA (15 mg/BW); RPRX group: received ROT (2.5 mg/BW) and pramipexole (PRX) (1 mg/BW).

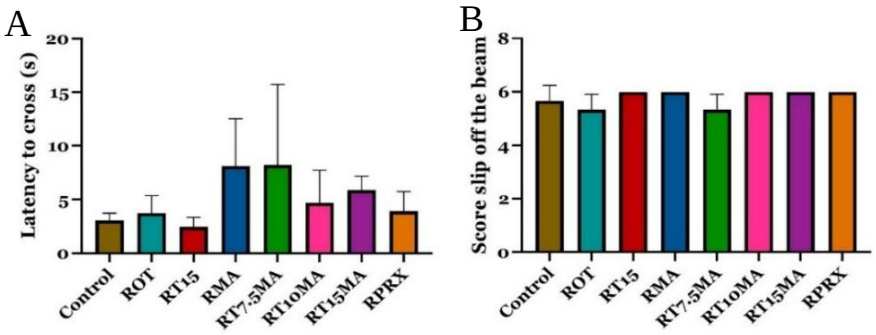

Figure 7. Effect of with thymoquinone (TQ) and madecassoside (MA) on beam walking test in (ROT)-induced mice: (A) traversal time on beam test, and (B) scoring in slip off the beam. The results are reported as mean±SD and evaluated using one-way ANOVA. Control group: received no treatment; ROT group: received ROT only (2.5 mg/BW); RT15 group: received ROT (2.5mg/BW) and TQ (15 mg/BW); RMA group: received ROT (2.5 mg/BW) and MA (15 mg/BW); R7.5MA group: received ROT (2.5 mg/BW),TQ (7.5mg/BW) and MA (15mg/BW); RT10MA group: received ROT (2.5 mg/BW), TQ (10 mg/BW), and MA (15 mg/BW); RT15MA group: received ROT (2.5 mg/BW), TQ (15 mg/BW), and MA (15 mg/BW); RPRX group: received ROT (2.5 mg/BW) and pramipexole (PRX) (1 mg/BW).

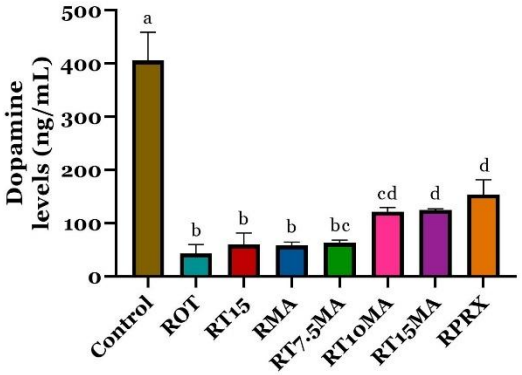

Figure 8. Effect of thymoquinone (TQ) and madecassoside (MA) on dopamine concentration in the midbrain in (ROT)-induced mice. Concentrations are expressed as mean±SD and analyzed using one-way ANOVA followed by Tukey's HSD post hoc test. Significant differences between groups were indicated by different letters on the top of the bars (p<0.05). Control group: received no treatment; ROT group: received ROT only (2.5 mg/BW); RT15 group: received ROT (2.5mg/BW) and TQ (15 mg/BW); RMA group: received ROT (2.5 mg/BW) and MA (15 mg/BW); R7.5MA group: received ROT (2.5 mg/BW),TQ (7.5mg/BW) and MA (15mg/BW); RT10MA group: received ROT (2.5 mg/BW), TQ (10 mg/BW), and MA (15 mg/BW); RT15MA group: received ROT (2.5 mg/BW), TQ (15 mg/BW), and MA (15 mg/BW); RPRX group: received ROT (2.5 mg/BW) and pramipexole (PRX) (1 mg/BW).

Effect of thymoquinone and madecassoside on mBDNF expression in cerebral cortex of ROT-induced mice

To examine the expression of mBDNF, immunohistochemical analysis was performed. The mBDNF antibody used in this study specifically recognized mBDNF and did not cross-react with pro-BDNF. mBDNF-positive cells were detected in all groups (**Figure 10A–H**), with localization observed in both the cytoplasm and nucleus of neurons. The percentage of mBDNF-positive cells was highest in the ROT group (49.8%–51%), indicating moderate to intense expression, compared to the control group (19.6%) and all treatment groups: RT15 (19.0%), RMA (19.2%), RT7.5MA (12.6%), RT10MA (23.4%), RT15MA (16.4%), and RPRX (29.6%). The percentage of mBDNF-positive cells in the control and treatment groups did not differ significantly from one another, but all were significantly lower than in the ROT group (p<0.05). The elevated expression of mBDNF in the cerebral cortex of ROT-exposed mice (**Figure 10I**) suggests a compensatory response to neurotoxicity, which warrants further investigation.

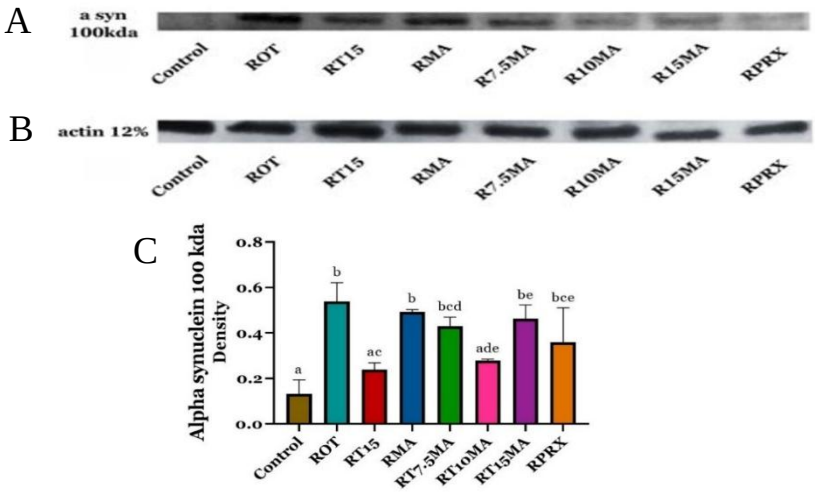

Figure 9. Effect of thymoquinone (TQ) and madecassoside (MA) on protein α-synuclein expression. (A) Expression of α-synuclein in 100kDa using Western blot. (B) Actin expression 12% as control using Western blot in (ROT)-induced mice. (C) Density of α-synuclein in all groups. Control group: received no treatment; ROT group: received ROT only (2.5 mg/BW); RT15 group: received ROT (2.5mg/BW) and TQ (15 mg/BW); RMA group: received ROT (2.5 mg/BW) and MA (15 mg/BW); R7.5MA group: received ROT (2.5 mg/BW), TQ (7.5mg/BW) and MA (15mg/BW); RT10MA group: received ROT (2.5 mg/BW), TQ (10 mg/BW), and MA (15 mg/BW); RT15MA group: received ROT (2.5 mg/BW), TQ (15 mg/BW), and MA (15 mg/BW); RPRX group: received ROT (2.5 mg/BW) and pramipexole (PRX) (1 mg/BW).

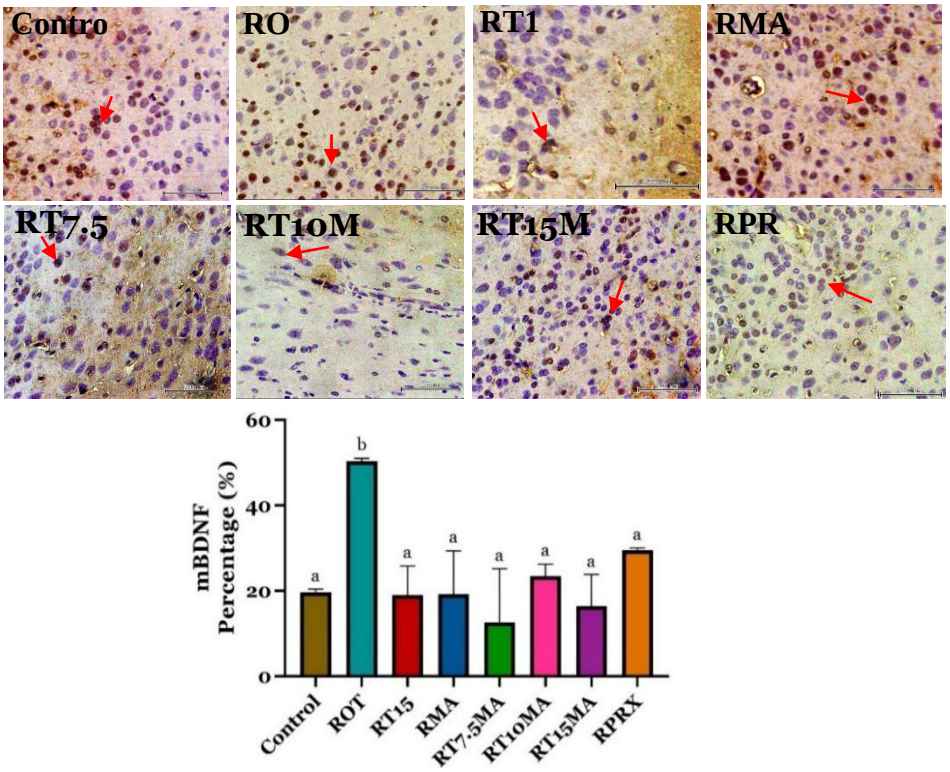

Figure 10. Effect of thymoquinone and madecassoside on mBDNF expression in cerebral cortex of ROT-induced mice. Immunoreactivity of mBDNF on cerebral cortex was indicated by a red arrow. Diaminobenzidine staining (brownish) showed positive reactivity of mBDNF. The graph showed the percentages of positive cells of mBDNF. The values are expressed as mean±SD and analyzed using one-way ANOVA followed by Tukey's HSD post hoc test. Significant differences between groups were indicated by different letters on the top of the bars ($p<0.05$). Control group: received no treatment; ROT group: received ROT only (2.5 mg/BW); RT15 group: received ROT (2.5mg/BW) and TQ (15 mg/BW); RMA group: received ROT (2.5 mg/BW) and MA (15 mg/BW); R7.5MA group: received ROT (2.5 mg/BW), TQ (7.5mg/BW) and MA (15mg/BW); RT10MA group: received ROT (2.5 mg/BW), TQ (10 mg/BW), and MA (15 mg/BW); RT15MA group: received ROT (2.5 mg/BW), TQ (15 mg/BW), and MA (15 mg/BW); RPRX group: received ROT (2.5 mg/BW) and pramipexole (PRX) (1 mg/BW).

Discussion

Parkinson's disease is a neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons, motor dysfunction, and impaired neuroplasticity [16]. The cardinal motor symptoms—bradykinesia, resting tremor, muscular rigidity, and postural instability—typically emerge after approximately 40–60% of dopaminergic neurons have degenerated and 30–40% of striatal dopamine content has been depleted [1]. In the present study, a mouse model of early Parkinson's disease was established using ROT administered at a dose of 2.5 mg/kg body weight every 48 hours for 16 days. This model was validated by a significant reduction in midbrain dopamine levels and a concomitant decline in motor performance, as evidenced by decreased total distance traveled and reduced mean velocity following ROT administration.

These findings are consistent with a previous study in which Swiss mice receiving subcutaneous ROT injections (1.5 mg/kg every 48 hours for two weeks) developed hypoactivity and motor symptoms resembling those observed in patients with Parkinson's disease [16]. Behavioral assessments are widely employed to evaluate Parkinsonian symptoms in animal models due to their simplicity and non-invasive nature [16]. The early detection of motor impairments remains critical for the preclinical evaluation of potential therapeutic agents [1]. Although ROT has been reported to cause non-specific effects, such as significant reductions in body weight, no significant changes in body weight were observed in this study either before or after treatment. These results are in agreement with a study reporting that subcutaneous administration of ROT (2.5 mg/kg every 48 hours) did not induce non-specific effects on body weight [18].

This study demonstrated that higher doses of the combined TQ and MA treatment significantly increased midbrain dopamine levels in ROT-induced mice, highlighting the potential of TQ and MA—particularly in combination—to mitigate motor and biochemical deficits in ROT-induced models of Parkinson's disease. Previous studies have reported that TQ possesses neuroprotective, anti-inflammatory, anti-cancer, and antioxidant properties. Its antioxidant activity has been shown to protect primary dopaminergic neurons from ROT and 1-methyl-4-phenylpyridinium (MPP^+)-induced toxicity, preserving tyrosine hydroxylase (TH)-immunoreactive cells [39,40]. Similarly, a study demonstrated that MA alleviated locomotor dysfunction and preserved dopaminergic neurons in MPTP-induced mouse models [30]. Notably, administration of TQ or MA alone failed to elevate dopamine levels, whereas their combination produced a significant increase. To the best of our knowledge, this is the first study to report the synergistic effect of TQ and MA in enhancing dopamine levels. These findings underscore the partial efficacy of the combined treatment—particularly at higher doses—in ameliorating dopamine depletion. Collectively, the results suggest that TQ and MA exert a neuroprotective effect on midbrain dopaminergic systems. Interestingly, motor coordination and muscle strength in the ROT group were not significantly different from the control or other treatment groups.

ROT-induced dopamine depletion has been associated with the cessation of locomotor activity [16]. This finding is consistent with the present study, which demonstrated a significant reduction in dopamine levels in the ROT group, accompanied by a marked decrease in locomotor performance, as indicated by reduced total distance traveled and mean velocity. Furthermore, administration of TQ appeared to mitigate movement impairment in ROT-induced mice, consistent with a previous study that reported the alleviation of movement deficits when TQ was combined with ROT during motor performance assessments [39]. Despite these improvements, motor coordination and muscle strength in the ROT group did not differ significantly from the control or treatment groups. Locomotor deficits have previously been linked to ROT-induced dopamine depletion [16], and ROT-induced Parkinson's disease models in rodents—regardless of dosage—have been shown to produce varying degrees of chronological, behavioral, and neurological impairments [18].

In ROT-induced mice, increased free radical production has been observed to accelerate α-synuclein aggregation. The abnormal accumulation and aggregation of α-synuclein in neuronal cell bodies and neurites are hallmark features of synucleinopathies, including Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy [41,42]. In this study, α-synuclein expression at 100 kDa appeared reduced in the treatment groups compared to the ROT

group; the differences were statistically significant in TQ group (15mg/BW) and the combination TQ (10mg/BW) and MA (15mg/BW) group. In contrast, dopamine levels differed significantly between the treatment and ROT groups. This discrepancy may reflect the early stage of Parkinson's disease modeled in this study, as the young age of the mice (8–10 weeks) may influence the vulnerability of dopaminergic neurons to α-synuclein aggregation. Age-dependent susceptibility to α-synuclein toxicity has been previously reported. Novel therapeutic strategies continue to focus on targeting pathological mechanisms involving α-synuclein fibrils, monomers, and oligomers, which are considered promising targets for modifying disease progression [43]. This study showed that co-administration of TQ and MA reduced α-synuclein expression while increasing dopamine levels, consistent with reports suggesting that α-synuclein regulates dopamine synthesis and signaling, including through dopamine receptor D2 (DRD2) pathways [44]. Moreover, α-synuclein has been shown to interact with tyrosine hydroxylase and the dopamine transporter, thereby influencing dopamine biosynthesis and catecholamine storage in synaptic vesicles [20]. A previous study also demonstrated the neuroprotective potential of TQ by showing that it delays α-synuclein aggregation and seeding in vitro, thereby preventing cellular toxicity [45].

mBDNF promotes the growth and survival of dopaminergic synapses in the substantia nigra, and inhibition of mBDNF function has been shown to lead to dopaminergic neuronal loss. In a mouse model of Parkinson's disease, reduced mBDNF expression has been associated with altered dopamine output in the corpus striatum, independent of dopaminergic activity in the substantia nigra. In the present study, the highest mBDNF expression in the cerebral cortex was observed in the ROT group. These findings are consistent with a previous study in which C57BL/6 mice administered intraperitoneal ROT (2.5 mg/kg/day for one week) exhibited a significant upregulation of neurotrophic factors, including BDNF and GDNF, in the brain [46]. This increase was proposed to underlie the lack of characteristic Parkinsonian features in short-term ROT models. In the current study, co-administration of TQ and MA (T10MA) in ROT-induced mice resulted in a higher percentage of mBDNF-positive cells compared to the control and other treatment groups, although the difference was not statistically significant.

TQ, an active compound in *Nigella sativa*, has demonstrated neuroprotective and anti-inflammatory effects, including increasing hippocampal BDNF levels and reducing hippocampal IL-6 and TNF-α levels. Similarly, MA, a triterpenoid compound found in *Centella asiatica*, contributes to the biological activity of the plant extract. A previous study reported that chronic administration of *C. asiatica* extracts enhanced total BDNF transcription, particularly in the prefrontal cortex, and significantly elevated mBDNF protein levels at a dose of 100 mg/kg. The combination of TQ (10 mg/kg) and MA (15 mg/kg) in the present study demonstrated a synergistic effect on increasing mBDNF expression in the cortex of ROT-induced mice. Further research is warranted to elucidate the mechanisms underlying this potential synergy and to validate its therapeutic relevance in Parkinson's disease.

mBDNF promotes the growth and survival of dopaminergic synapses in the substantia nigra. Inhibition of mBDNF function has been associated with dopaminergic neuronal loss. In a mouse model of Parkinson's disease, reduced mBDNF expression has been shown to alter dopamine output in the corpus striatum, independent of dopaminergic activity in the substantia nigra. In the present study, the highest mBDNF expression in the cerebral cortex was observed in the ROT group. These findings are consistent with a previous study using C57BL/6 mice treated with intraperitoneal ROT (2.5 mg/kg/day for one week), which reported a significant increase in neurotrophic factors, including BDNF and glial cell-derived neurotrophic factor (GDNF), in the brain [46]. This upregulation may explain the failure of ROT to induce classical Parkinsonian features in short-term models.

Administration of a combination of TQ and MA (T10MA) to ROT-induced mice resulted in an increased percentage of mBDNF-positive cells compared to the control and other treatment groups, although the difference was not statistically significant. TQ, an active compound derived from *Nigella sativa*, has demonstrated neuroprotective and anti-inflammatory properties, including the ability to enhance hippocampal BDNF expression and reduce IL-6 and TNF-α levels in the hippocampus [47]. MA, a triterpenoid compound found in *Centella asiatica*, has also been shown to modulate neurotrophic signaling. A previous study reported that chronic

administration of *C. asiatica* extract increased total BDNF transcription, particularly in the prefrontal cortex, and significantly elevated mBDNF protein levels following treatment with 100 mg/kg of the extract [48]. In the current study, the combination of TQ (10 mg/kg) and MA (15 mg/kg) demonstrated a synergistic effect in elevating mBDNF expression in the cortex of ROT-induced mice. Further research is warranted to confirm this synergy and elucidate the underlying mechanisms involved in their anti-parkinsonian effects.

Conclusion

Parkinson's disease is a progressive neurodegenerative condition for which no effective treatment currently exists. TQ, the main active compound of *Nigella sativa*, and MA, the main active compound of *Centella asiatica*, have been identified as promising neuroprotective agents as anti-parkinsonian. The combination of TQ and MA dose-dependent significantly elevated dopamine levels, decreased the expression of α-synuclein aggregation, and thereby, improving locomotor function in ROT-induced mice in early Parkinson's disease.

Ethics approval

Animal handling and experimental design were carried out in line with the Research Ethical Committee regulations at the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia (Code Number: 178/EC/KEPCONTROLS3/06/2024).

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

Declaration of artificial intelligence use

This study used artificial intelligence (AI) tools and methodologies in the following capacities. AI-based language model, ChatGPT, was employed to language refinement and technical writing assistance. We confirm that all AI-assisted processes were critically reviewed by the authors to ensure the integrity and reliability of the results. The final decisions and interpretations presented in this article were solely made by the author.

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