

Mercury-induced neurotoxicity in rat: in vivo and in silico studies of thymoquinone modulatory effect via oxidative stress and inflammation, HO-1/NRF/Trx/Trx-R trajectories

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Mercury-induced neurotoxicity in rat: in vivo and in silico studies of thymoquinone modulatory effect via oxidative stress and inflammation, HO-1/NRF/Trx/Trx-R trajectories

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Abstract

Mercury exposure has been associated with the onset of neurodegenerative disorders via mechanisms that include oxidative stress, inflammation, and apoptosis. Thymoquinone (TQ) has been documented to possess antioxidant, anti-inflammatory, and apoptotic modulatory effects. We assessed TQ's neuroprotective potential against mercuric chloride (HgCl₂) induced neurotoxicity in rats. Experimental groups included: Control; HgCl₂ alone (20 µg/mL); TQ alone (5 mg/kg); and co-administration of HgCl₂ with TQ at doses of 2.5 or 5 mg/kg over a period of 28 days. Behavioural changes were evaluated on days 27 and 28 using the open field, negative geotaxis, and forelimb grip tests. Subsequently, oxidative stress, lipid peroxidation, inflammation, and apoptosis were measured in the cerebral and cerebellar cortices; histology and histomorphometry were also performed. Alone, HgCl₂ reduced locomotor and exploratory activity and induced anxiety-like behaviour. Co-treatment with TQ significantly ($p < 0.05$) improved these behaviours. HgCl₂ decreased ($p < 0.05$) cerebral and cerebellar antioxidant levels while increasing ($p < 0.05$) oxidative/nitrosative stress. TQ co-treatment increased antioxidant activities and dose-dependently reduced ($p < 0.05$) pro-oxidant levels. TQ co-treatment reversed ($p < 0.05$) the HgCl₂-induced changes in inflammatory, pro-apoptotic, and anti-inflammatory biomarkers. Histomorphometry revealed that TQ reduced ($p < 0.05$) HgCl₂-induced neuronal pyknosis and loss of pyramidal and Purkinje cells. TQ interacts with PPAR-γ (Molecular docking Score: 1.65×10^{-5} M), enhances Nrf-2/HO-1 antioxidant signalling and downregulates NF-κB inflammatory pathways. TQ protects against HgCl₂-induced neuronal toxicity by boosting antioxidant defences, reducing oxidative and inflammatory stress through Nrf-2/HO-1 and NF-κB pathways, and involving PPAR-γ signalling.

Keywords Thymoquinone, Mercuric chloride, Neurobehavioural, Antioxidant, Anti-inflammatory, Apoptosis

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Introduction

Neurodegenerative diseases involve the gradual loss and death of neurons in the brain and spinal cord (Gadhav et al., 2024 [1]). Although the exact cause of these disorders is still unknown, they are influenced by genetic, endogenous, and environmental factors—including toxicants such as mercury (Brown et al., 2005; Lamprey et al., 2022 [2]). Mercury, a heavy metal, has recommended safety limits for water, soil, and food (Aleku et al., 2024 [3]; Wu et al., 2024 [4]). Nevertheless, ongoing human activities in agriculture, industry, and medicine mean exposure to mercury remains common [5]. Mercury exists in several forms: elemental (metallic) mercury and vapour (Hg_0), inorganic mercury (such as mercuric chloride, HgCl_2), and organic mercury (methylmercury, MeHg) (Bernhoft, 2012 [6]; Souza-Rodrigues et al., [7]; [5]). These forms can interconvert, allowing mercury to cross the blood–brain barrier with a lethal dose of 1–4 g (Beasley et al., 2014 [8]). Mercury toxicity affects various organs, including the liver, kidneys, brain, heart, gastrointestinal tract, and reproductive system, resulting in neurotoxicity, hepatotoxicity, nephrotoxicity, gastrointestinal toxicity, and reproductive toxicity (Said et al., 2021 [9]; [5]). Research shows mercury is associated with an imbalance in pro-oxidant and antioxidant systems, marked by excess ROS production, reduced antioxidant activity, mitochondrial dysfunction, neuroinflammation, and apoptosis (Ajsuvakova et al., 2020 [10]; Pizzino et al., 2017 [11]; Sabir et al., 2022 [12]), which can lead to neurodegenerative disorders and neuronal cell death (Said et al., 2021). Mercury exposure leads to dopamine neuron loss in the substantia nigra, causes beta-amyloid and neurofibrillary tangle formation, triggers phosphorylated tau protein, memory loss, and promotes alpha-synuclein aggregation into Lewy bodies (Bjorklund et al., 2018 [13]; Sibling et al., 2019 [14]). Mercury toxicity is linked to calcium imbalance, disrupted glutamatergic transmission, oxidative stress, and neuroinflammation [15, 16].

Recent research highlights medicinal plants as alternatives to conventional drugs. *Nigella sativa* (black cumin or black seed), a member of the Ranunculaceae family, has demonstrated significant medicinal potential (Darakhshan et al., 2015). Thymoquinone (TQ), the primary bioactive compound in the volatile oil of *Nigella sativa* seeds (Woo et al., 2012), demonstrates a wide range of pharmacological effects, including anti-oxidant, anti-inflammatory, anti-hypoglycemic, anti-hypertensive, anti-asthmatic, anti-neoplastic, anti-histaminic, anti-microbial, anti-tumour, and immunomodulatory activities in both in vitro and in vivo studies (Banerjee et al., 2010 [17]; Darakhshan et al., 2015 [18]; Gali-Muhtasib et al., 2006; [19] [20, 21]). TQ has been utilised for conditions such as neurodegenerative disorders, coronary artery diseases, and respiratory and urinary diseases.

Additionally, Alrafiah [22] reports that TQ supplementation can prevent neuronal damage, increase serum antioxidant enzymes, and decrease inflammatory cytokine levels after a high-fat diet. TQ has been associated with nephroprotection against mercury chloride (HgCl_2) in rats by modulating enzymes and protein levels, such as SOD, CAT, GSH, Nrf-2, Hsp-70, and Bcl-2, and reducing caspase-3 activity (Sabir et al., 2022). However, current data are insufficient to fully clarify the mechanisms underlying TQ's protective effects against mercury-induced neurodegeneration. This study aims to investigate whether TQ can protect against HgCl_2 -induced neurotoxicity in rats through behavioural, biochemical, histological, and molecular docking approaches.

Materials and methods

Chemicals

Mercury Chloride (HgCl_2 ; $\geq 99.5\%$, 7487–94-7), Thymoquinone (TQ; $\geq 98\%$, 490–91-5), thiobarbituric acid (TBA, 504–17-6), 5,5-dithio-bis-2-nitrobenzoic acid (DTNB, 69–78-3), trichloroacetic acid (TCA; 76–03–9), Hydrogen peroxide (H_2O_2 ; 7722–84-1), Epinephrine (51–42-3), and CDNB –1-chloro-2,4-dinitrobenzene (97–00–7) procured from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Rat's Enzyme-Linked Immunosorbent Assay ELISA-plates was used for the microplate estimation of caspase 3 (E-EL-R0160), caspase 9 (E-EL-R0163), Interleukin-10 (IL-10; E-EL-R0016), Nuclear Factor, Erythroid Derived 2, (NRF2; E-EL-R1052), Heme Oxygenase 1 (HO-1; E-EL-R0488), Thioredoxin (Trx; E-EL-H1728), Thioredoxin reductase (Trx-R; E-EL-H1727) activities supplied by Elabscience Biotech, Wuhan, China.

Experimental animals care

Forty male albino Wistar rats (150–200 g), purchased from the Experimental Animal House, Faculty of Veterinary Medicine, Ibadan, Nigeria, were used for this investigation. The experimental rats were housed in rodent cages and kept in a well-ventilated animal house. The rats were provided with standard rat pellets (Ladokun™ Feeds, Ibadan, Nigeria) and had unrestricted access to clean drinking water. Rats were acclimated for one-week preceding experimentation under a 12-h light: 12-h dark cycle photoperiod. Experimental animals were sufficiently cared for, as detailed in the “Guide for the Care and Use of Laboratory Animals” published by the National Institute of Health [23]. Additionally, experimental protocols were approved by the Animal Welfare and Ethical Use of Laboratory Animal Committee at the University of Ibadan (UI-ACUREC/057–1222/11).

Experimental design

The rats were randomly grouped into five treatment groups of eight rats each and were treated for 28

uninterrupted days, as depicted in Fig. 1 and illustrated below:

- Group 1: Control: received distilled water (2 mL/kg).
- Group 2: HgCl₂ (20 µg/L).
- Group 3: TQ (5 mg/kg p.o) only.
- Group 4: HgCl₂ (20 µg/L) and TQ1 (2.5 mg/kg).
- Group 5: HgCl₂ (20 µg/L) and TQ2 (5.0 mg/kg).

The doses of Thymoquinone (TQ) were administered via oral gavage [24]. Mercury Chloride (HgCl₂; 20 µg/L) was made available in drinking water with a standard estimated dose ranging 0.36–0.48 µg/day of 200 rats, obtained via an average water intake of 9–12 mL per 100 g body weight/day. The stock solution was prepared by dissolving 1 g of HgCl₂ in 1 L of distilled water to result a yield 1 mg/mL. 20 µL of stock was further diluted in 1 L to yield 20 µg/L provided ad libitum to HgCl₂ treated experiment rats for 28 days [20, 21].

Behavioural tests

The open field test was used to assess the locomotive and exploratory behaviour of rats in all groups, which were monitored on Day 28 in a novel wooden box

(100×100×60 cm) following the method of Owumi [25]. Briefly, each rat was placed in the centre of the box sterilized with 70% ethanol and cotton wool to remove olfactory cue and allowed to roam freely for 10 min. At the same time, the rat's behaviour was captured by a webcam (DNE webcam) directly situated overhead the novel box. The videotapes were analysed of 5 min cut-off using video-tracking software (ANY-maze, Stoelting Co). Automatically generated locomotive and explorative behavioral parameters were average speed, total distance travelled, path efficiency, absolute turn angle, frequency of rearing and grooming, time spent in the centre and immobility time. A negative geotaxis test was performed to assess motor fitness, and the time taken to re-orient on a 45° incline plane was noted. In contrast, a forelimb grip test was conducted by suspending the forepaws on a horizontal wire and recording the time it took for the experimental animals to fall from the wire, thereby determining the strength of their forelimbs [26].

Neurochemical assay

After the behavioural assessment, the rats were weighed, anaesthetised using ketamine (50 mg/kg) and killed. The brain was carefully removed and separated into the

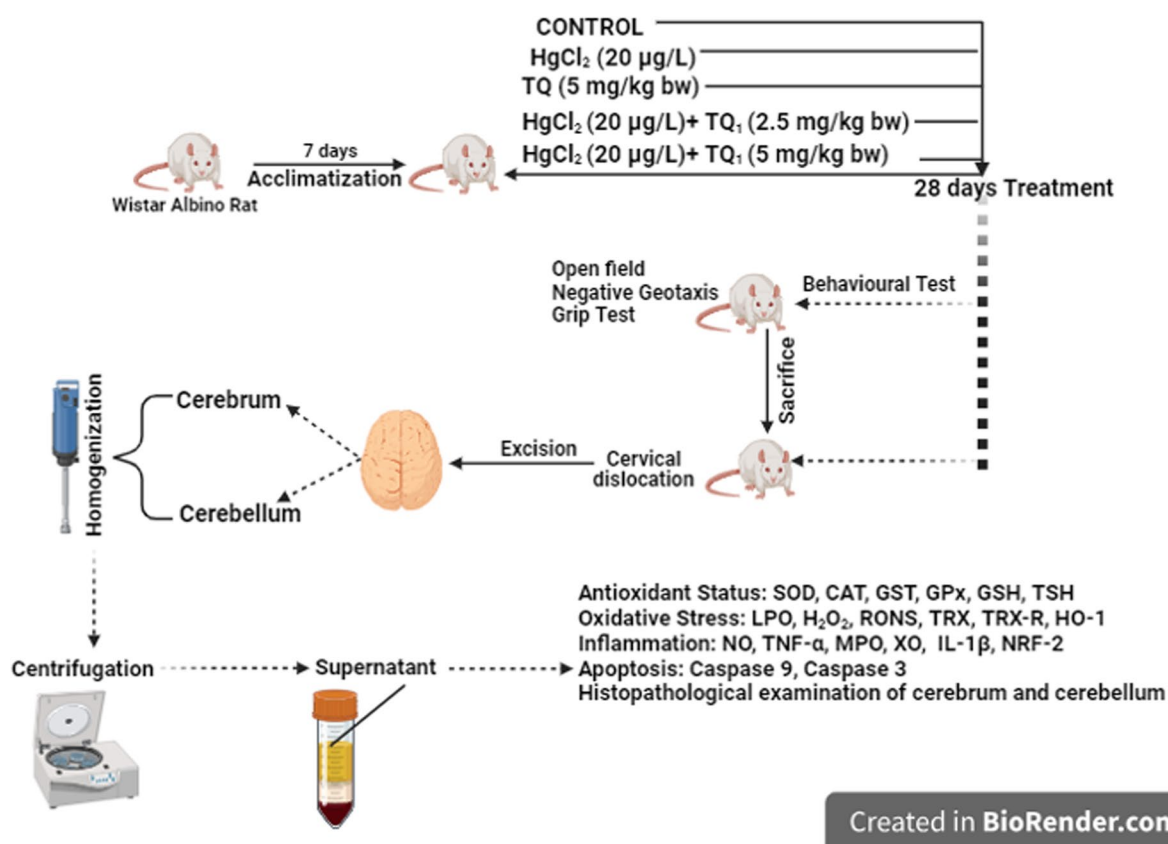


Fig. 1 A—B Outlines of the experimental timeline to investigate the effect of TQ on HgCl₂-induced neurotoxicity in rats, Albino Wistar strain, for 28 uninterrupted days (A). Created by Uche Arunsi using <https://app.biorender.com/>. Control: distilled water; TQ: Thymoquinone; HgCl₂: Mercury Chloride

cerebrum and cerebellum before being homogenised in ten volumes of 50 mM Tris–HCl buffer (pH 7.4). Subsequently, the homogenate was centrifuged (12,000 g at 4 °C for 15 min), and the supernatants from the cerebrum and cerebellum were used for biochemical assays. Activities of antioxidant enzymes, namely catalase (CAT) activity in hydrogen peroxide (19 mM) every minute for 5 min as substrate at 240 nm [27], superoxide dismutase (SOD) was assessed by monitoring the autoxidation of adrenochrome in the presence of superoxide and read at 480 nm [28], glutathione peroxidase (GPx) in sodium azide (10 mM), hydrogen peroxide (2.5 mM), and GSH (4 mM) at 412 nm following [29], glutathione-S-transferase (GST) in 1-chloro-2,4-dinitrobenzene (CDNB; 20 mM) and GSH (1 M) reagent as co-substrate every 60 s in 3 min at 340 nm [30]. The level of glutathione (GSH) was assayed, as described by Jollow [31], using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), which produced a yellow colour that was measured at 412 nm, and total sulfhydryl activity (TSH), in Ellman's reagent [32] at 412 nm. The activity of xanthine oxidase (XO) in xanthine solution (0.15 mM) was read every minute for 3 min at 290 nm in line with the standard procedure of Bergmeyer et al. [33]. The levels of lipid peroxidation (LPO) in thiobarbituric acid reactive substances (TBARS) and reactive oxygen and nitrogen species (RONS) in 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were evaluated as previously described [34, 35], respectively. Inflammatory indices, namely nitric oxide (NO) level in the Griess reagent and myeloperoxidase activity in hydrogen peroxide complex reaction with o-dianisidine to produce a yellow product at 460 nm, were assessed using standard procedures by Green et al. [36] and Granell et al. [37], respectively. Besides, anti-inflammatory activity (interleukin 10, (IL-10), nuclear factor erythroid 2-like 2, NRF2, heme oxygenase-1, HO-1, thioredoxin Trx, thioredoxin reductase, Trx-R, as well as caspase-3 and 9 activities were analysed using ELISA Kits as itemised in the manufacturer's guide. Briefly, 100 µL of the standard working solution provided by the manufacturer, blank and supernatant samples (cerebral and cerebellar), were added to 96 wells containing IL-10, NRF2, HO-1, Trx, Trx-R, casp-3, and casp-9. Each plate and liquid content were sealed and incubated at 37 °C for 90 min. Then, the fluid contents were decanted without being washed, and 100 µL of biotinylated detection Ab working solution was added to each well, gently mixed, sealed, and incubated for 1 h at 37 °C. The solution in each plate was decanted and washed with 350 µL of wash buffer, followed by a 1–2 min soak and drying against clean absorbent paper three times. Then, 90 µL of substrate reagent was added to each well, the wells were sealed, and the samples were incubated for 15 min at 37 °C in a dark environment. The reaction was then stopped by adding

50 µL of stop solution to each well. The OD value was measured immediately at 450 nm within 15 min using an M384 SpectraMax Multimodal plate reader (Molecular Devices, San Jose, CA, USA). The intensity of colour developed directly relates to the concentration of IL-10, NRF2, HO-1, Trx, Trx-R, Casp-3 and Casp-9 in the brain sample.

Histological and histomorphometric evaluation

Histological and histomorphometric assessments of the cerebellum and cerebrum were performed using 5-µm sections stained with hematoxylin and eosin, following the standard protocol [38]. Purkinje and cortical neurons were quantified by counting the viable and degenerated neurons using a graticule and a microscope at varied magnifications. A minimum of 10 observations was charted in each slide, and the average values were computed for each group. A Pathologist identified viable neurons at high power by distinct nucleoli, dispersed chromatin, and the absence of cell death features, specifically karyolysis and pyknosis. The number of degenerated Purkinje and cortical neurons was presented.

Molecular docking of thymoquinone with selected proteins

The results from the in vivo studies were supported by observations from in silico studies through molecular docking simulations. Peroxisome proliferator-activated receptor gamma (PPAR-γ) is a well-established therapeutic target that regulates antioxidant defences, inflammation, and apoptosis. Several PPAR-γ agonists have demonstrated clinical efficacy in managing metabolic and inflammatory disorders [39–42]. Therefore, we selected PPAR-γ as the molecular docking target to evaluate thymoquinone's potential to modulate these cytoprotective pathways, complementing the in vivo neuroprotective effects observed in our study. In this research, we investigated the interaction between TQ and PPAR-γ and quantified the binding constant (Kd) using molecular docking scores. For this purpose, the 3-D structures of TQ were downloaded from PubChem: <https://pubchem.ncbi.nlm.nih.gov>, while the structures of the proteins PPAR-γ (PDB: 5TGN) were obtained from the Protein Database (PDB): <https://www.rcsb.org/>. UCSF ChimeraX [43] was utilised to prepare the proteins. Additionally, the ligand (TQ) from the synthesised receptors was loaded into PyRx, and Open Babel was employed for energy minimisation. Following this, molecular docking was conducted using grid boxes designed around the position of the co-crystallised ligand. The docking modes that exhibited the lowest Gibbs free energy were then visualised with Ligplot and PyMol. The binding constant (Kd) was estimated from the general information $\Delta G = -RT \ln K_d$, where R:

Table 1 Body weight gain and relative Cerebrum and Cerebellum weight of rats following exposure to HgCl₂ for 28 days

Total rats per grouping	Control	HgCl ₂	TQ	HgCl ₂ + TQ1	HgCl ₂ + TQ2
Body weight (g)	219.50 ± 8.73	219.20 ± 16.33	217.20 ± 30.86	223.5 ± 12.01	240.2 ± 13.44
Body weight gain (g)	60.86 ± 8.07	57.00 ± 12.45	61.29 ± 14.20	65.25 ± 10.26	72.63 ± 16.18
Cerebrum weight (g)	0.99 ± 0.15	0.68 ± 0.23	0.82 ± 0.12	0.88 ± 0.20	0.80 ± 0.27
Relative Cerebrum weight (%)	0.45 ± 0.07	0.31 ± 0.10	0.39 ± 0.11	0.39 ± 0.07	0.33 ± 0.12
Cerebellum weight (g)	0.26 ± 0.02	0.21 ± 0.04	0.24 ± 0.03	0.24 ± 0.02	0.26 ± 0.01
Relative Cerebellum weight (%)	0.12 ± 0.01	0.14 ± 0.11	0.11 ± 0.03	0.09 ± 0.02	0.10 ± 0.02

HgCl₂ (20 µg/L dissolved in drinking water); TQ1 and TQ2 (2.5 and 5.0 mg/Kg respectively) body weight; *n* = 6. Data are expressed as mean ± SD. HgCl₂: Mercuric chloride; TQ: Thymoquinone

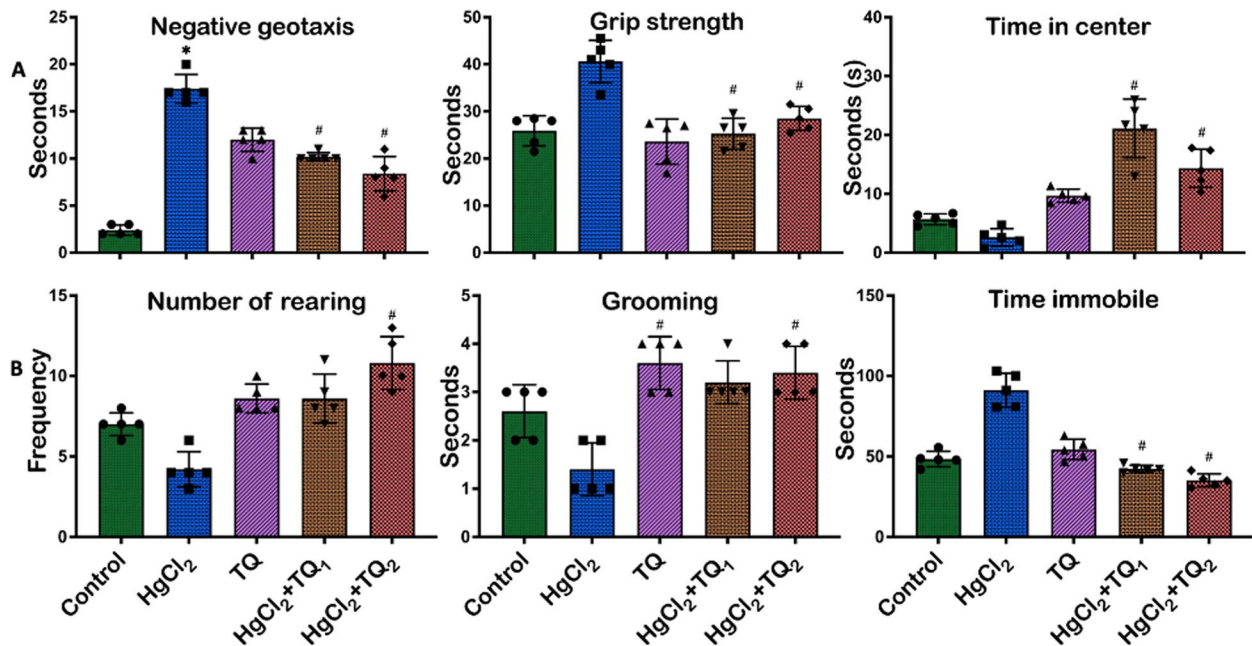


Fig. 2 The effect of TQ on HgCl₂-induced anxiety-related (A) and motor dysfunction (B) in male Wistar rats experimentally treated for 28 consecutive days. Each bar represents mean ± SD (*n* = 5). The statistics were analysed using GraphPad Prism V.10.2 for macOS. The tests used were the Kruskal–Wallis and Dunn's post hoc test. The designated *P*-values for all tests were set at **p* < 0.05 vs Control, #*p* < 0.05 vs HgCl₂ alone. Control, distilled water; TQ: Thymoquinone; HgCl₂: Mercury Chloride

Gas constant = 0.001987 kcal/mol/K, *T*: Temperature in Kelvin = 298 K, Δ*G*: Gibbs free energy.

Statistical analyses

All data are presented as mean ± SD and were analyzed using GraphPad Prism V.10.2 for macOS. Shapiro–Wilk normality and lognormality tests were performed followed by Kruskal–Wallis and Dunn's post hoc test for multiple comparisons to detect significant difference between experimental groups considering *p* < 0.05 statistically significant.

Results

TQ reversed the HgCl₂-induced reduction in body and organ weight of animals

Table 1 depicts the effect of TQ on the body weight, organ and relative organ weight of HgCl₂-treated experimental rats. Compared with the control group, there was

no significant difference in the body weight (*p* < 0.05), cerebrum weight (*p* < 0.05), cerebellum weight (*p* < 0.05), relative weights of the cerebrum (*p* < 0.05) and cerebellum (*p* < 0.05) of experimental animals treated with HgCl₂ alone. However, co-treatment with TQ (2.5 and 5 mg/kg) exhibited no significant difference in the body weight, cerebrum and cerebellum weight and their respective relative weight compared with HgCl₂ alone administration rats.

TQ abated HgCl₂-induced anxiogenic-like behaviour and motor activity decline in rats

The effects of TQ on anxiogenic-like behaviour and motor activity dysfunction in HgCl₂-treated rats are depicted in Fig. 2. In comparison with the control, Kruskal–Wallis and Dunn's test yielded a significant increase in the time to re-orient on the inclined plane (*p* < 0.05), the latency to fall (*p* < 0.05) on the forelimb grip test,

no significant reduction in the time spent in the center, rearing time, and grooming time and an increase in the immobility time on open field test in HgCl₂ administration experimental rats. Conversely, co-treatment with TQ (2.5 and 5 mg/kg) significantly ($p < 0.05$) reduced the time to re-orient on the inclined plane and inactivity to fall on the forelimb grip test, while, significantly reversing anxiogenic behaviours by increasing the number of rearing, grooming, time spent in center and decreasing immobility time in the OFT compared to HgCl₂ treated rats.

TQ reversed HgCl₂-induced impairment of exploratory and locomotor actions in rats

As illustrated in Figs. 3, Kruskal–Wallis and Dunn's test indicated significant different among treated groups in locomotive and explorative activities include total distance travelled ($P < 0.05$), absolute turn angle ($P < 0.05$), path efficiency ($P < 0.05$), line crossing ($P < 0.05$), average speed ($P < 0.05$). Compared with the control group, HgCl₂-treated rats exhibited locomotor activity dysfunction, as shown by decreases in total distance travelled, absolute turn angle, grooming time, path efficiency, line crossing, average speed, and trajectory plots. However, the TQ co-treated group (2.5 and 5 mg/kg) enhanced locomotion and exploratory activities by increasing total distance travelled, absolute turn angle, path efficiency, line crossing, average speed, and trajectory plots,

and lowering heat map intensity compared to the HgCl₂ group.

TQ modulated HgCl₂-induced decrease in antioxidant status

Kruskal–Wallis and Dunn's test indicated significant different ($P < 0.05$) among treated groups in the antioxidant status on cerebrum and cerebellar cortices (upper panel, lower panel) including superoxide dismutase (SOD), catalase (CAT), glutathione-s-transferase (GST) as presented in Fig. 4–5. HgCl₂-treated rats exhibited a decrease in both the activities of cerebral and cerebellar cortices SOD, CAT, GST (Fig. 4), GPx, GSH, and TSH (Fig. 5), respectively, when compared with the control. On the other hand, TQ co-treatment (2.5 and 5 mg/kg) significantly ($P < 0.05$) increases the antioxidant status compared to the HgCl₂-treated group in a dose-dependent manner.

TQ mitigate HgCl₂-induced increase in RONS, LPO and XO activities

Kruskal–Wallis and Dunn's test yielded significant different ($P < 0.05$) among treated groups in the pro-oxidant status on cerebrum and cerebellar cortices (upper panel, lower panel) is presented in Fig. 6. The results showed that cerebro-cerebellar activity of RONS, LPO and XO where higher in the HgCl₂ alone treated groups ($p < 0.05$) when compared to control. Meanwhile, co-treatment

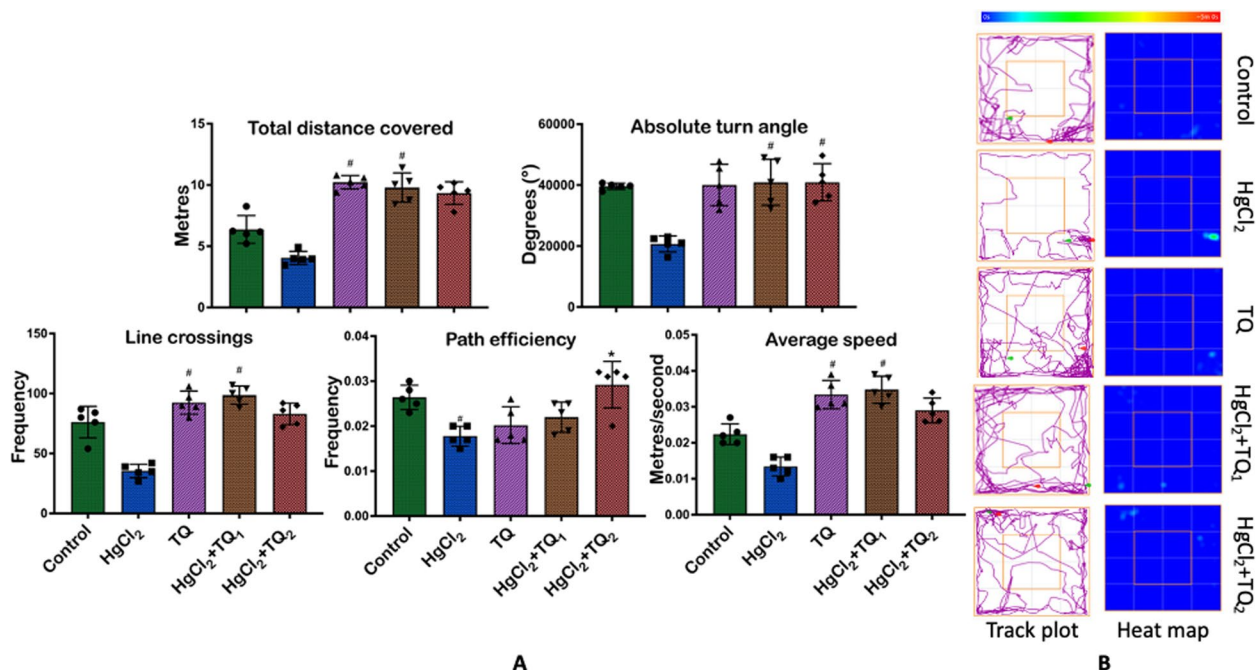


Fig. 3 The effect of TQ on HgCl₂-induced locomotive decline (A) and explorative behaviour (B) in male Wistar rats treated for 28 consecutive days. Each bar represents mean $\pm$ SD ($n = 5$). The statistics were analysed using GraphPad Prism V.10.2 for macOS. The tests used were the Kruskal–Wallis and Dunn's post hoc test. The designated P-values for all tests were set at * $p < 0.05$ vs Control, # $p < 0.05$ vs HgCl₂ alone. Control: distilled water; TQ: Thymoquinone; HgCl₂: Mercury Chloride

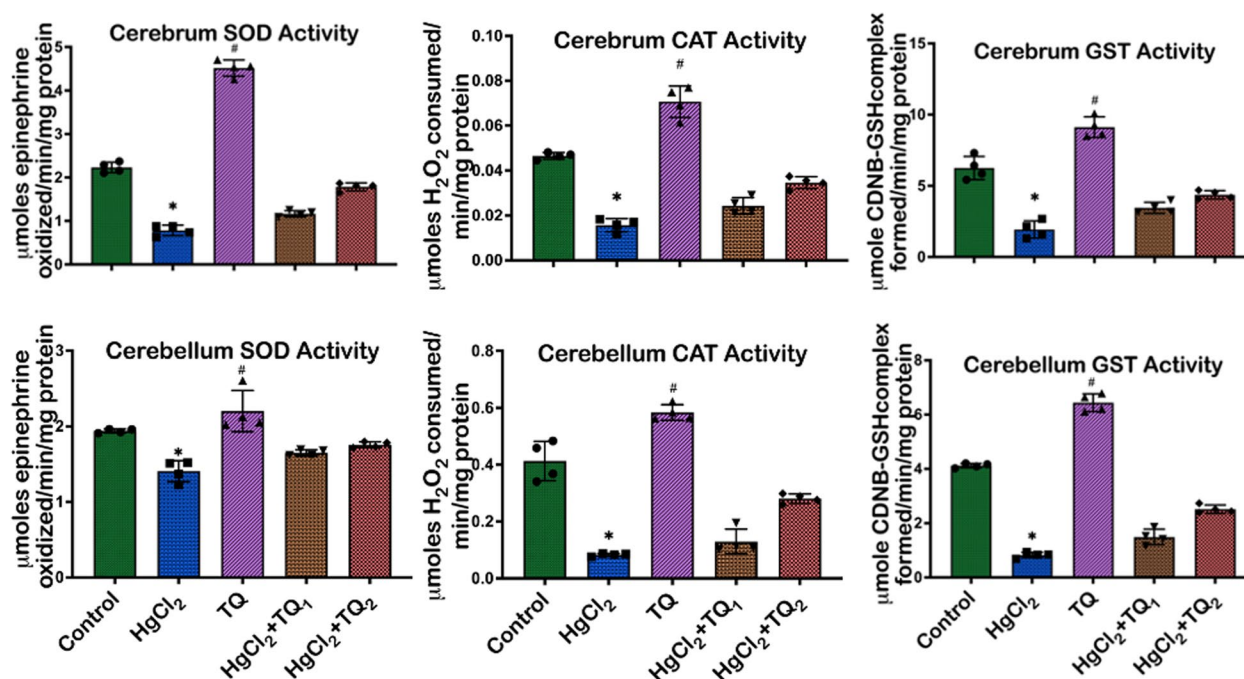


Fig. 4 The influence of TQ on SOD, CAT and GST activities in the cerebrum and cerebellum of HgCl_2 -treated rats for 28 consecutive days. Each bar represents mean $\pm$ SD ($n=5$). The statistics were analysed using GraphPad Prism V.10.2 for macOS. The tests used were the Kruskal–Wallis and Dunn's post hoc test. The designated P-values for all tests were set at $*p < 0.05$ vs Control, $\#p < 0.05$ vs HgCl_2 alone. Control: distilled water; TQ: Thymoquinone; HgCl_2 : Mercury Chloride. SOD: Superoxide dismutase; CAT: Catalase; GST: Glutathione S-transferase

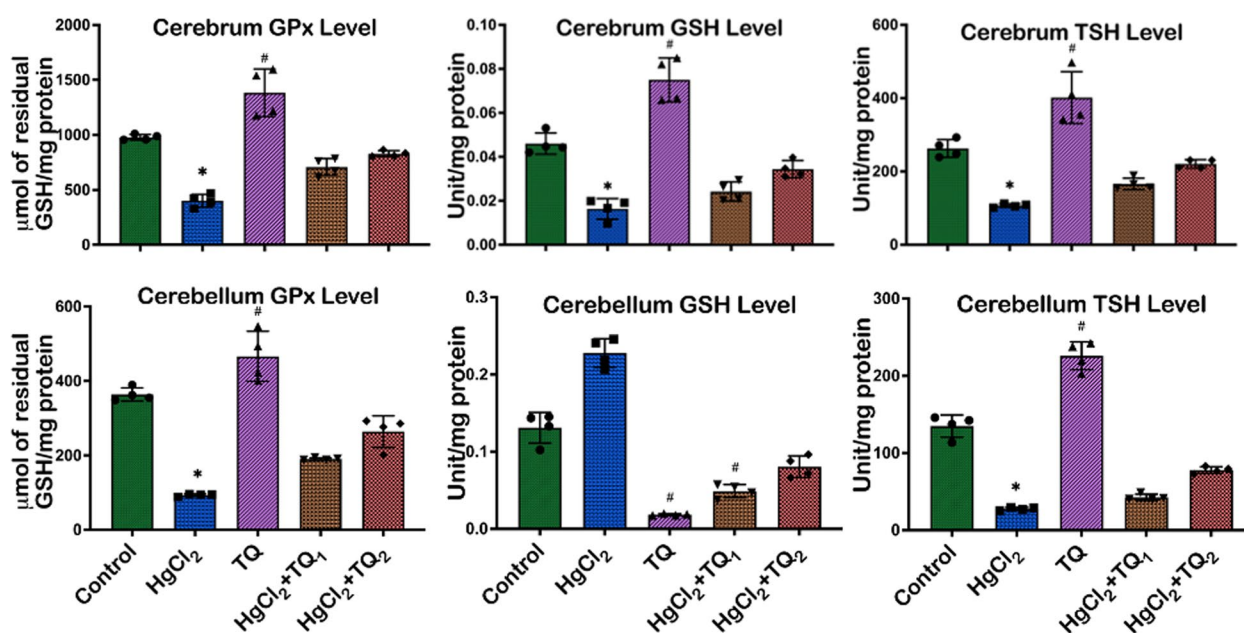


Fig. 5 The influence of TQ on GPx activity, GSH, and TSH levels in the cerebrum and cerebellum of HgCl_2 -treated rats for 28 consecutive days. Each bar represents mean $\pm$ SD ($n=5$). The statistics were analysed using GraphPad Prism V.10.2 for macOS. The tests used were the Kruskal–Wallis and Dunn's post hoc test. The designated P-values for all tests were set at $*p < 0.05$ vs Control, $\#p < 0.05$ vs HgCl_2 alone. Control: distilled water; TQ: Thymoquinone; HgCl_2 : Mercury Chloride. GPx: Glutathione peroxidase, GSH: Glutathione; TSH: Total sulfhydryl group

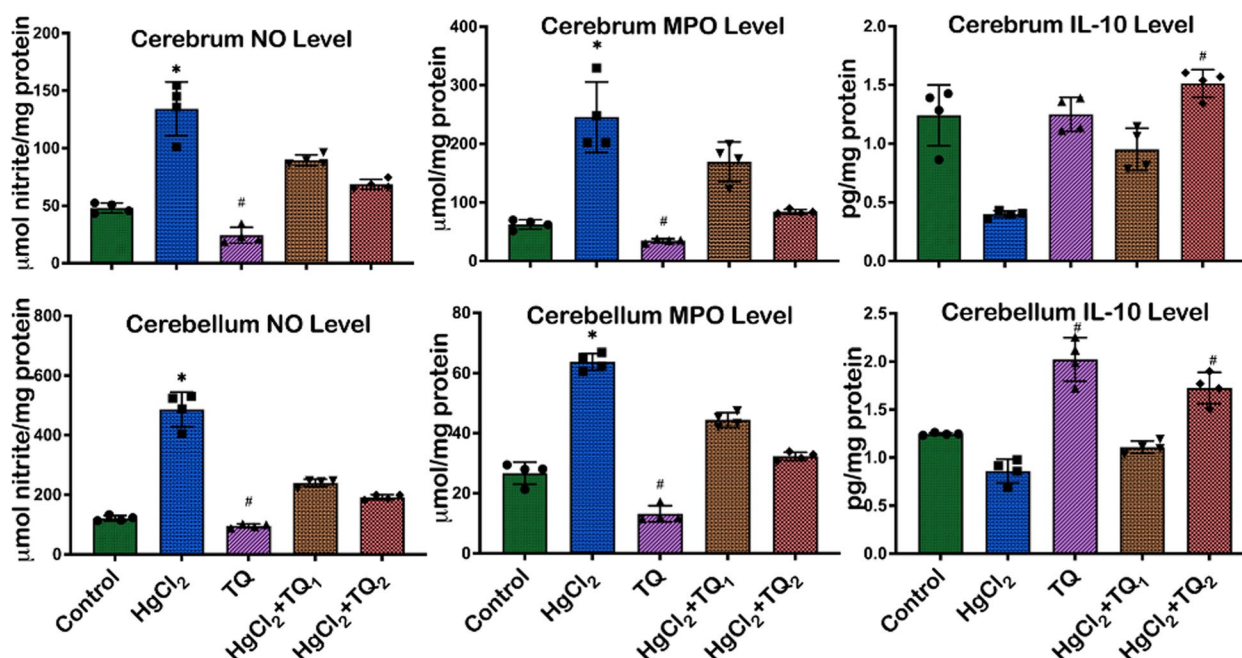


Fig. 6 The influence of TQ on NO level, MPO activity, and IL-10 levels in the cerebrum and cerebellum of HgCl₂-treated rats for 28 consecutive days. All value represents the mean ($n=4$; $\pm$ SEM). The statistics were analysed using GraphPad Prism V.10.2 for macOS. The tests used were Kruskal–Wallis and Dunn's post hoc test. The designated P-values for all tests were set at * $p < 0.05$ vs Control, # $p < 0.05$ vs HgCl₂ alone. Control: distilled water; TQ: Thymoquinone; HgCl₂: Mercury Chloride. NO: Nitric oxide; MPO: Myeloperoxidase; TNF- α : Tumour necrosis factor-alpha

with low and high doses of TQ reversed HgCl₂-alone induced increase in the cerebral and cerebellar pro-oxidant by decreasing RONS, XO, and LPO levels in brain sections.

TQ reversed HgCl₂-induced inflammation mediators

The effect of TQ on HgCl₂-induced inflammatory molecules and cytokines is depicted in Fig. 7. Compared to the control group, Kruskal–Wallis and Dunn's test showed that HgCl₂-treated rats exhibited a significant ($p < 0.05$) increase in inflammatory molecules including the levels of nitric oxide (NO), myeloperoxidase (MPO) and decreased anti-inflammatory cytokines interleukin 10 (IL-10) in both the cerebral and cerebellar cortices. However, co-treatment with TQ (2.5 and 5 mg/kg) reversed the effect of HgCl₂-alone treatment in experimental rats by reducing cerebral and cerebellar inflammatory molecules and upregulating anti-inflammatory cytokines.

TQ upregulated HgCl₂-induced increase in the cytoprotective enzyme system and antioxidant signalling molecule

The effect of TQ on cerebrocerebellar cytoprotective enzyme system of HgCl₂-treated rats is depicted in Figs. 8 and 9. Compared to the control group, Kruskal–Wallis and Dunn's test yielded a significant reduction ($P < 0.05$) in the cerebral and cerebellar cortices activities of thioredoxin (Trx), thioredoxin reductase (Trx-R) Fig. 8, along with antioxidant signaling molecules nuclear factor

erythroid 2 related factor 2 (Nrf-2), and heme oxygenase-1 (HO-1) Fig. 9, of HgCl₂-treated rats. On the other hand, co-treatment with graded doses of TQ (2.5 and 5 mg/kg) restored Trx and Trx-R levels and enhanced Nrf-2 and HO-1 levels compared to the HgCl₂-treated group.

TQ suppressed HgCl₂-induced increase in apoptosis biomarkers in the cerebral and cerebellar brain sections of rats

Figure 10 shows Kruskal–Wallis and Dunn's test revealed significant difference ($P < 0.05$) in the cerebrocerebellar apoptotic biomarkers among group treated with TQ and HgCl₂. Compared with the control group, experimental rats exposed to HgCl₂ alone significantly ($P < 0.05$) increased the activities of Caspase-3 and Caspase-9 in cerebral and cerebellar cortices (Fig. 10). In contrast, co-treatment with TQ (2.5 and 5 mg/kg) downregulated cerebrocerebellar apoptotic activities typified by the marked reduction in caspases 3 and 9 activities as compared to rat cohorts treated with HgCl₂ alone.

TQ abrogated histomorphometric and histological modifications in HgCl₂-exposed rats

The impact of TQ on cerebrocerebellar histological and histomorphometry examination of rats treated with HgCl₂ is depicted in Figs. 11 and 12. Rats exposed to HgCl₂ alone significantly ($P < 0.05$) reduced in number of viable cerebral pyramidal cell and cerebellar Purkinje

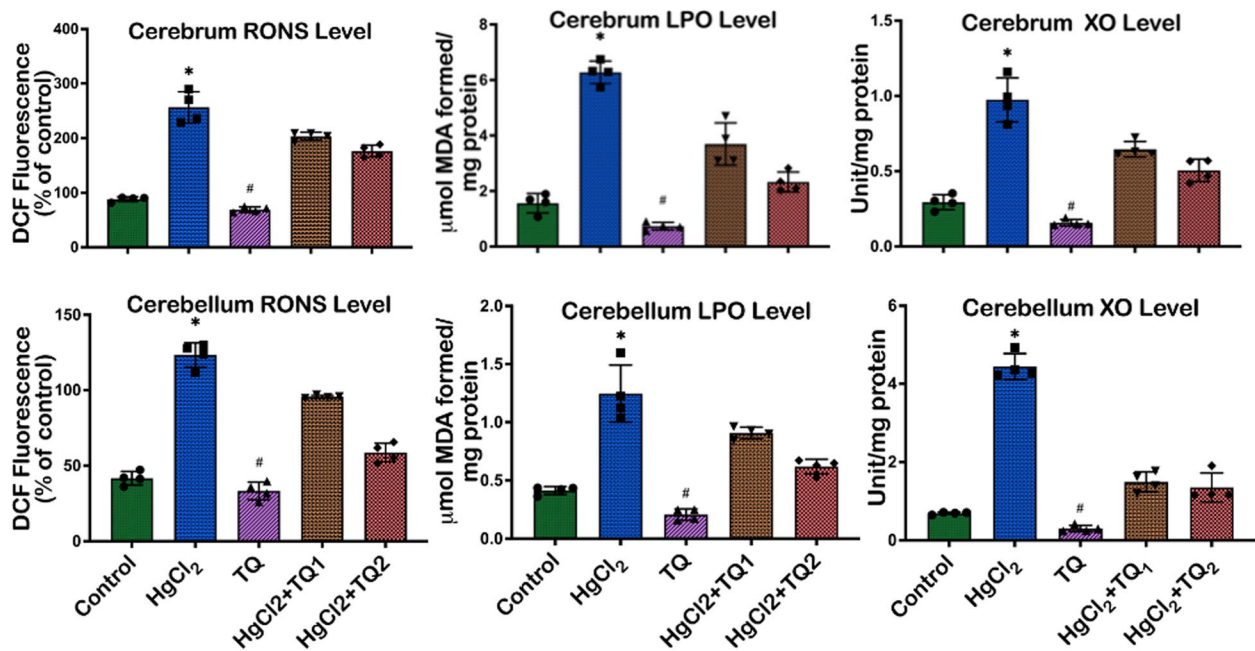


Fig. 7 The influence of TQ on RONS, LPO levels, and XO activity in the cerebrum and cerebellum of HgCl₂-treated rats for 28 consecutive days. Each bar represents mean $\pm$ SD ($n=5$). The statistics were analysed using GraphPad Prism V.10.2 for macOS. The tests used were the Kruskal–Wallis and Dunn's post hoc test. The designated P-values for all tests were set at * $p < 0.05$ vs Control, # $p < 0.05$ vs HgCl₂ alone. Control: distilled water; TQ: Thymoquinone; HgCl₂: Mercury Chloride. RONS: Reactive oxygen and nitrogen species; XO: Xanthine oxidase; LPO: Lipid peroxidation; and PC: Protein carbonyl

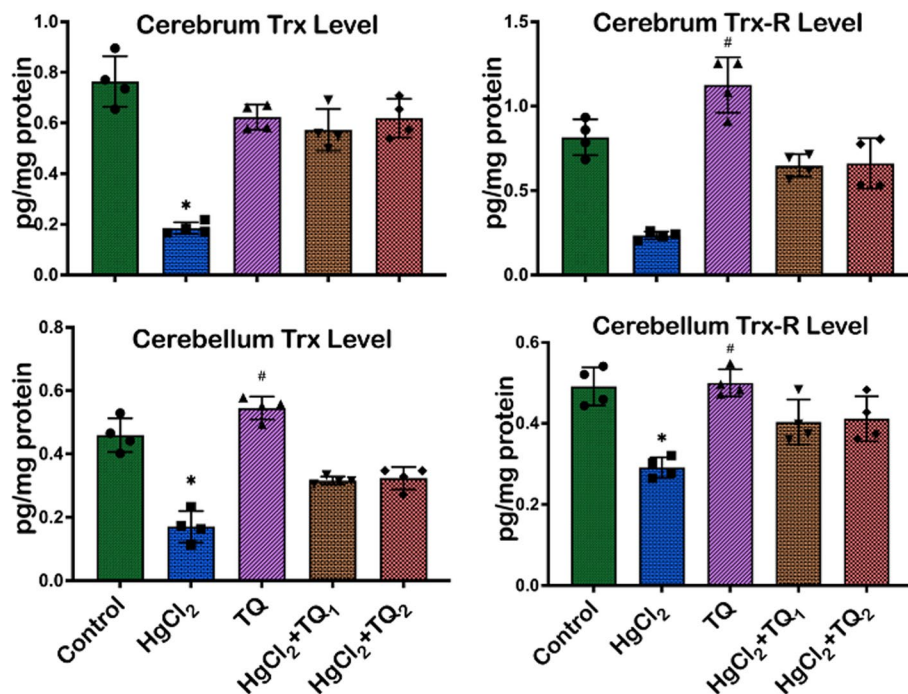


Fig. 8 The influence of TQ on Trx and Trx-R in the cerebrum and cerebellum of HgCl₂-treated rats for 28 consecutive days. Each bar represents mean $\pm$ SD ($n=4$). The statistics were analysed using GraphPad Prism V.10.2 for macOS. The tests used were the Kruskal–Wallis and Dunn's post hoc test. The designated P-values for all tests were set at * $p < 0.05$ vs Control, # $p < 0.05$ vs HgCl₂ alone. Control: distilled water; TQ: Thymoquinone; HgCl₂: Mercury Chloride. Trx: thioredoxin; Trx-R thioredoxin reductase

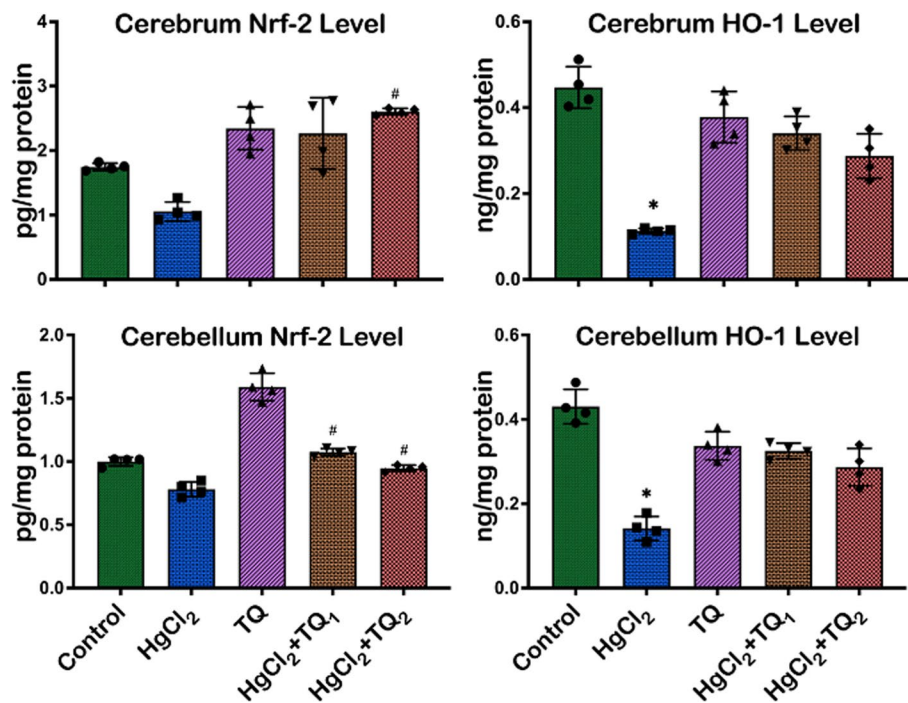


Fig. 9 The effect of TQ on HgCl₂-induced alterations in Nrf-2 and HO-1 signalling molecules in the cerebrum and cerebellum of rats treated for 28 consecutive days. Each bar represents mean $\pm$ SD ($n=4$). The statistics were analysed using GraphPad Prism V.10.2 for macOS. The tests used were Kruskal–Wallis and Dunn’s post hoc test. The designated P-values for all tests were set at * $p < 0.05$ vs Control, # $p < 0.05$ vs HgCl₂ alone. Control: distilled water; TQ: Thymoquinone; HgCl₂: Mercury Chloride. Nrf-2: nuclear factor, erythroid-derived 2; HO-1: Heme oxygenase-1

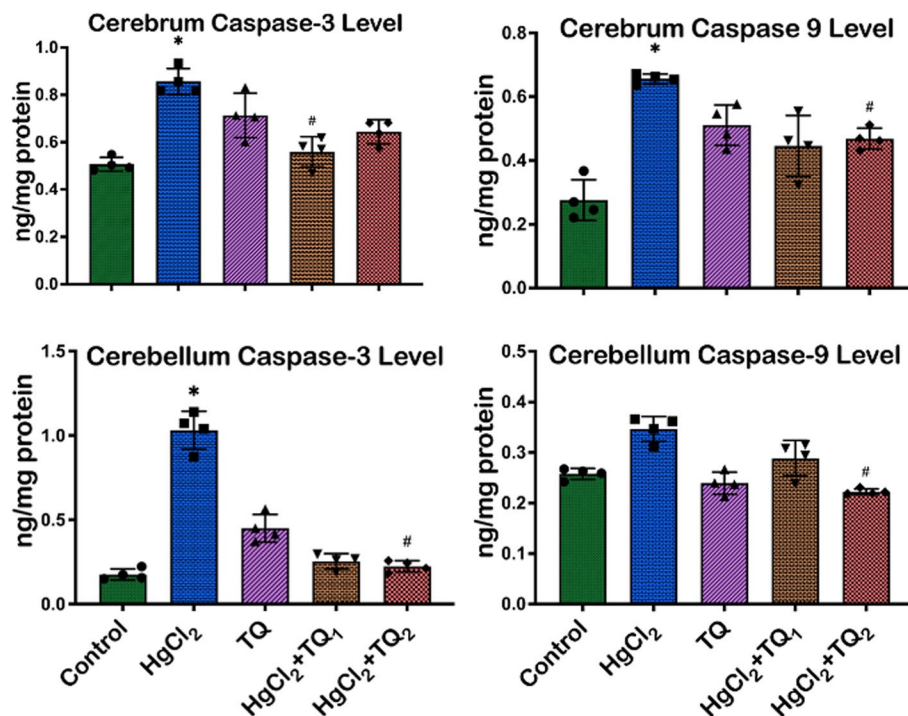


Fig. 10 The effect of TQ on apoptotic biomarkers -Caspase 3 and Caspase 9- in the cerebrum and cerebellum of HgCl₂-treated rats for 28 consecutive days. Each bar represents mean $\pm$ SD ($n=4$). The statistics were analysed using GraphPad Prism V.10.2 for macOS. The tests used were Kruskal–Wallis and Dunn’s post hoc test. The designated P-values for all tests were set at * $p < 0.05$ vs Control, # $p < 0.05$ vs HgCl₂ alone. Control: distilled water; TQ: Thymoquinone; HgCl₂: Mercury Chloride

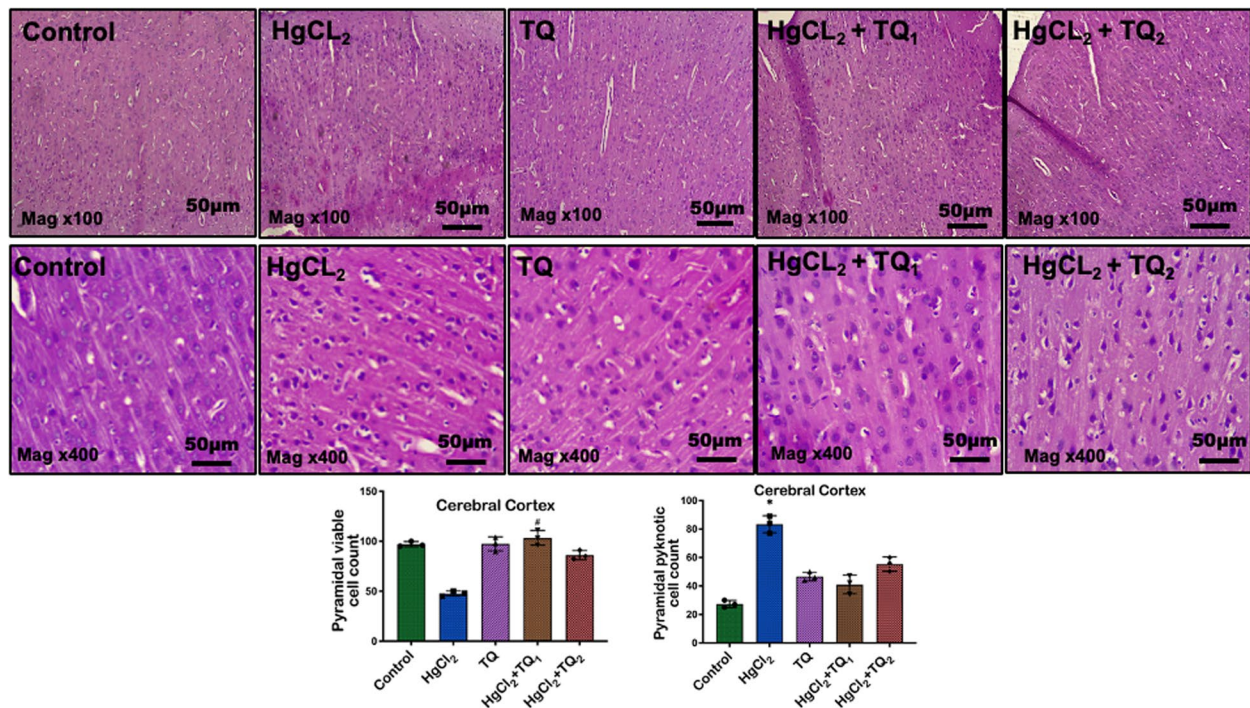


Fig. 11 Representative histological changes on the effect of TQ in the cerebral cortex of experimental rats treated with HgCl_2 for 28 consecutive days. Each bar represents mean $\pm$ SD ($n = 3$). The statistics were analysed using GraphPad Prism V.10.2 for macOS. The tests used were Kruskal–Wallis and Dunn's post hoc test. The designated P-values for all tests were set at $*p < 0.05$ vs Control, $^{\#}p < 0.05$ vs HgCl_2 alone. Control: distilled water; TQ: Thymoquinone; HgCl_2 : Mercury Chloride

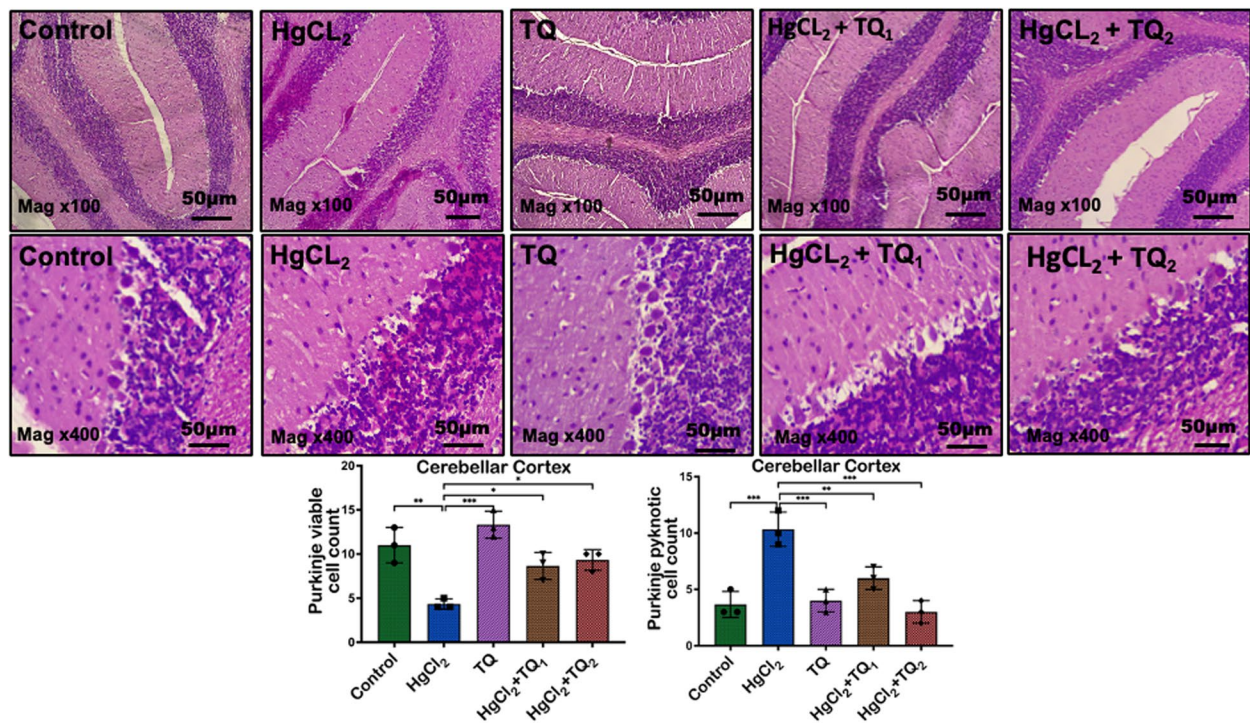


Fig. 12 Representative histological changes in the cerebellar cortex on the effect of TQ in rats treated with HgCl_2 for 28 consecutive days. Each bar represents mean $\pm$ SD ($n = 3$). The statistics were analysed using GraphPad Prism V.10.2 for macOS. The tests used were the Kruskal–Wallis and Dunn's post hoc test. The designated P-values for all tests were set at $*p < 0.05$ vs Control, $^{\#}p < 0.05$ vs HgCl_2 alone. Control: distilled water; TQ: Thymoquinone; HgCl_2 : Mercury Chloride

cell accompanied by marked increase in the number of pyknotic cells in the cerebral cortex and cerebellar cortex when compared with control. However, co-treatment with TQ (2.5 and 5 mg/kg) reversed HgCl₂-induced histopathological toxicity, evidenced by a significant reduction in cerebral and cerebellar cortical pyknotic cells, accompanied by a concomitant increase in the number of viable pyramidal and Purkinje cells, when compared to HgCl₂-only treated rats.

TQ mediate antioxidant, anti-inflammatory and anti-apoptotic activities through PPAR-gamma signalling

Following the findings from the *in vivo* studies, we conducted molecular docking simulations to explore TQ's potential to mediate antioxidant, anti-inflammatory, and anti-apoptotic effects. We assessed the binding constant (K_d) from TQ's interactions with PPAR-γ. The results showed that TQ had a moderate interaction with PPAR-γ, as demonstrated by a K_d value of 1.65×10^{-5} M (Fig. 13A). Through this interaction, PPAR-γ can positively engage Nrf-2 to induce antioxidant, anti-inflammatory, and anti-apoptotic effects, while negatively regulating NF-κB to abate the expression of pro-inflammatory mediators (Fig. 13B). Altogether, these results suggest that TQ may possess antioxidant, anti-inflammatory, and anti-apoptotic effects by agonistically activating PPAR-γ.

Discussion

The current study investigates the neuro-modulatory effect of thymoquinone on mercuric chloride-induced neurotoxicity through cerebro-cerebellar oxidative, inflammatory, cytoprotective, and apoptotic pathways,

involving the enzymes HO-1, Nrf-2, and Trx. Our study observed that rats exposed to HgCl₂ developed derangements, characterised by neurobehavioral alterations associated with impaired motor function and disrupted histological architecture in various brain regions, including the cerebral and cerebellar cortices. In agreement with our current results, Teixeira et al. [5] have reported that accumulations of mercury in several brain regions (cerebellum, temporal, and frontal lobes) at chronic exposure, despite its lower liposolubility, caused motor and cognitive deficits along with alterations in neurotransmitters. This agrees with our findings, which demonstrated a decrease in distance travelled, absolute turn angle, line crossing, path efficiency, average speed, trajectory plots, and grip strength, indicating motor dysfunction and an increase in anxiogenic activities suggested by a decrease in the time spent in the centre, grooming and rearing of the open field. Interestingly, TQ reversed motor impairment and anxiogenic behaviour caused by HgCl₂.

Further investigation revealed that TQ-related behavioural improvement may be mediated through oxidative/nitrosative stress, inflammation and apoptosis in the motor cortices (cerebral and cerebellar regions). According to previous reports, the motor cortex mediates higher-order cognitive-motor movements, sensorimotor integration, and fine-motor control [44, 45]. The motor cortex receives afferents from the prefrontal and parietal cortices and projects them to the thalamus, cerebellum, and spinal cord as the primary source of voluntary cortical motor commands. Notably, neuronal motion control originates from the prefrontal cortex and is modulated by the basal ganglia and cerebellum [45]. Therefore, injuries

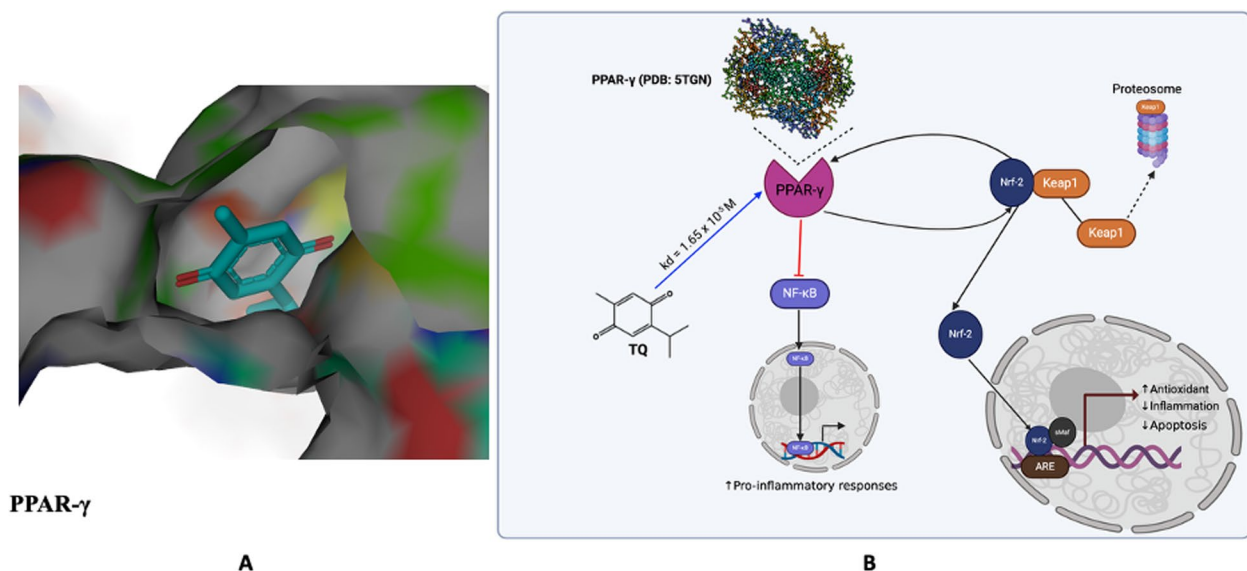


Fig. 13 Molecular docking illustration showing the binding of thymoquinone (TQ) with PPAR-γ (PDB: 5TGN) with ΔG values of -6.4 kcal/mol (A). TQ can activate PPAR-γ, which in turn represses NF-κB and activates Nrf-2, leading to the upregulation of the expression of cytoprotective factors and inhibition of inflammation and apoptosis (B)

in motor cortex regions disrupt the sensorimotor network, causing impairments in fine motor coordination [46]. These motor cortex injuries are linked to oxidative stress, indicated by increased production of ROS, which causes a decline in brain antioxidant activity, induced neuroinflammation, neurochemical imbalance, protein aggregation, and apoptosis [47, 48]. Exposure to HgCl_2 above the safe limit has been reported to alter the biological integrity of the brain barrier through its interaction with cellular mechanisms, including Na^+/K^+ ATPase and glutathione system, enhancing the metal responsive element (MRE) and expression of the metallothionein gene activation, promoting motor dysfunction and neurodegeneration [49]. This causes enzymatic Na^+/K^+ ATPase activity inhibition and subsequent increase in oxidative-nitrosative stress, lipid, protein and DNA peroxidation, antioxidant depletion, intracellular Ca^{2+} overload and mitochondrial dysfunction in the brain [15, 49–53]. Studies have reported that chronic exposure to HgCl_2 caused alterations in enzymatic activity of the antioxidant system in different brain regions, such as the cerebral cortex, olfactory bulb, hippocampus, hypothalamus, mesencephalon, pons, cerebellum, and medulla oblongata demonstrated by decrease activity of catalase, thioredoxin reductase, glutathione peroxidase, superoxide dismutase, and glutathione S-transferase, accompanied by peroxidation of membrane lipids, indicating a disturbance in intracellular redox homeostasis with subsequent increased intracellular oxidative stress [54–56] which may be due to high metabolic rate and increased oxygen demand in the brain tissue in response to stress [57]. Notably, exposure to HgCl_2 may elicit several mechanisms that lead to the generation of intracellular reactive oxygen and nitrogen species [56, 58, 59]. This is in agreement with our current study, as indicated by elevated levels of RONS nitric oxide (NO), lipid peroxidase (LPO), xanthine oxidase (XO), and the marked decrease in antioxidant enzymes GST, GPx, CAT, GSH, and SOD activities in cerebral and cerebellar cortices, which enhance cellular production of ROS in HgCl_2 -treated rats. The significant reduction in these antioxidant defences suggests that HgCl_2 -induced ROS generation may have overwhelmed the brain's protective mechanisms.

Our findings demonstrate that the neuroprotective effect of TQ was through upregulation of the antioxidant response system, including the enzymatic and non-enzymatic antioxidant system, thioredoxin system, Nrf-2 and the downregulation of inflammatory and apoptotic mediators as reported by previous literature [60–64]. TQ abated HgCl_2 -induced oxidative and inflammatory dysfunction by increasing the thioredoxin and glutathione system, downregulating pro-inflammatory cytokines and apoptotic biomarkers and enhancing the anti-inflammatory system. In agreement with our findings, Alrafiah

[22], S. [20, 21] suggested that the therapeutic potential of TQ is associated with its ability to reduce body weight, improve antioxidant enzyme activity, mitigate inflammatory mediators, and enhance anti-inflammatory effects in high-fat diet rat models. Our findings showed improved GSH and thioredoxin (Trx/Trx-R) systems, which are reported to maintain the intracellular redox status by scavenging ROS production in cells [16], regulating several signalling pathways through reversible disulfide bond formation in the target proteins [16, 65, 66]. Specifically, thioredoxin reductase (Trx-R) is essential for diverse cellular functions, including proliferation, maintenance of redox homeostasis, DNA synthesis, regulation of gene expression, and control of cell death by apoptosis and represents an important target for diseases associated with oxidative stress [67]. GSH is a non-enzymatic antioxidant that defends against exogenous toxic injury by augmenting the defence against ROS via scavenging of free radicals, whereas glutathione peroxidase (GPX) and catalase enhance enzymatic catalysis necessary for the elimination of hydroperoxides. TQ also exerts anti-inflammatory and antioxidant effects through molecular pathways by downregulating cytokine release and activating nuclear factor erythroid 2-related factor 2 (Nrf-2), phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT), and nuclear factor kappa-light-chain-enhancer of activated B (NF- κ B) [68]. This was typified by enhanced HO-1, Nrf2 and IL-10 levels in the co-treatment groups, suggesting neuroprotection of TQ.

In this study, we further provided evidence of the anti-apoptotic effects of TQ, as indicated by the reduction in cerebral and cerebellar concentrations of Casp-9 and Casp-3, which were upregulated in rats treated with HgCl_2 only. Converging studies have reported an increase in the tissue level of caspase-3 and caspase-9 expression, which serves as an essential indicator of toxic insult, thereby conferring cell death by activating apoptotic pathways [69–71]. Hence, it is not out of place to ascribe apoptotic response to HgCl_2 to the increase in caspase-3 and caspase-9 in the cerebrum and cerebellar brain tissue section. Our findings agreed with a previous study that reported that exposure to HgCl_2 elicits damaging effects on mitochondria in cerebellar granule cells [72] and causes apoptotic pathway activation in human lung fibroblast MRC5 cells by the upregulation of caspase-8 (extrinsic) and caspase-9 (intrinsic) and altered caspase-3 activity [73]. TQ co-administration reversed the increased number of apoptotic and pyknotic cells in the cerebral and cerebellar tissues of the HgCl_2 -only group, providing compelling evidence of TQ's anti-apoptotic effect and ability to reverse programmed cell death in these tissues. However, definitive histopathology markers were not examined. Peroxisome proliferator-activated receptors (PPARs) are an excellent therapeutic target

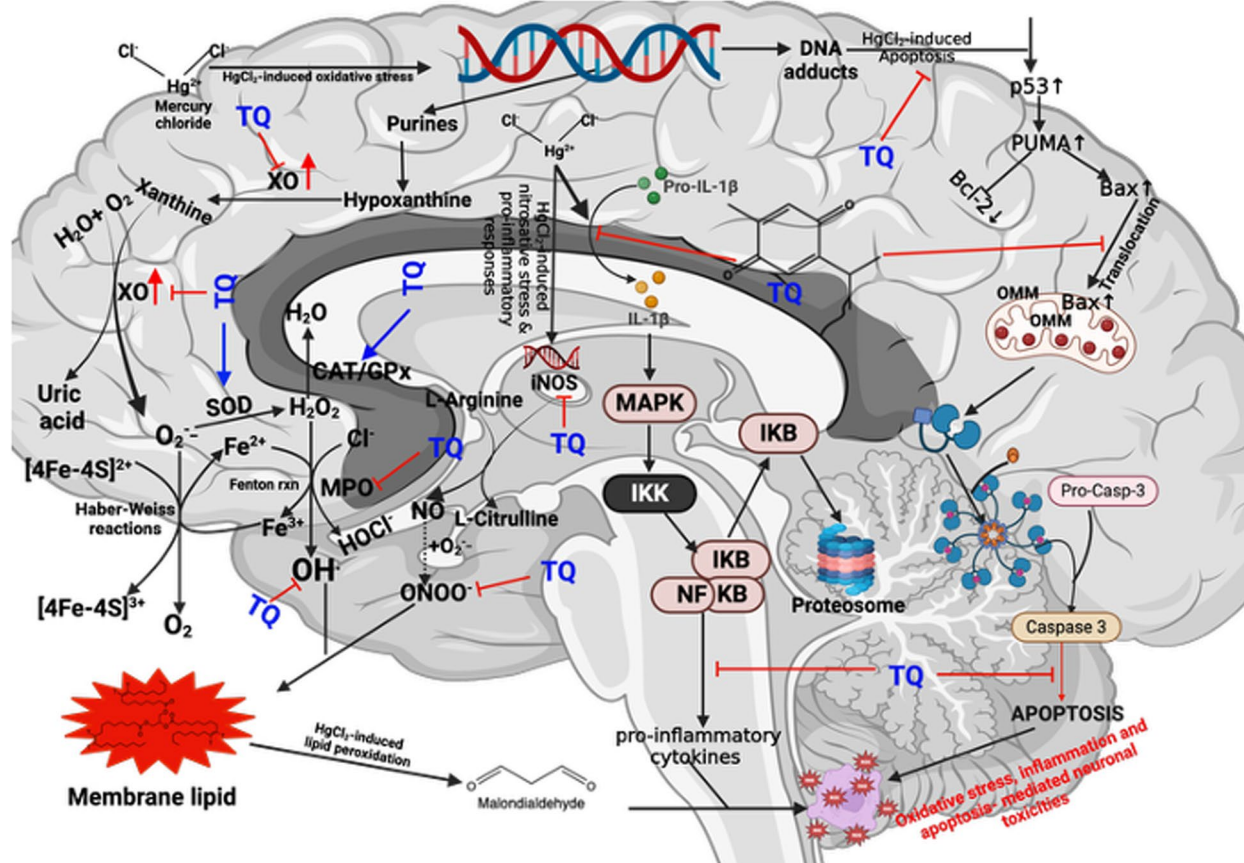


Fig. 14 Proposed mechanistic mode of action of TQ neuroprotective effect against HgCl₂-induced neurotoxicity in male Wistar rats. Created by Uche Arunsi using <https://app.biorender.com/>

for novel anti-diabetic lead molecules. PPAR- γ (PPAR- γ) has been proven experimentally to confer health benefits, including induction of antioxidant response, modulation of inflammation, and protection of programmed cell death [39–42]. Some agonists of PPARs have demonstrated considerable usefulness in clinical settings for managing metabolic diseases [74–76]. Using molecular docking principles, we evaluated the molecular interactions between TQ and PPAR- γ . The results from our in-silico studies demonstrate that TQ can agonistically interact with PPAR- γ . This may increase the potential of this protein to inhibit NF- κ B, while necessitating the translocation of Nrf-2 into the nucleus, where it binds to the antioxidant response element (ARE) to induce the expression of cytoprotective mediators.

Our findings indicate that TQ reduces HgCl₂-induced neuronal toxicity by boosting endogenous antioxidants to counteract ROS generated by HgCl₂. This process reduces pro-inflammatory mediators, apoptosis, and the histological structure of the cerebral and cerebellar cortices. SOD transforms superoxide anion radicals, produced by HgCl₂-induced oxidative DNA damage, into hydrogen peroxide. If not converted into water by CAT or GPx, the

generated hydrogen peroxide can react with labile irons ($\text{Fe}^{2+}/\text{Fe}^{3+}$) in the Fenton reaction to produce hydroxyl radicals, which are more toxic ROS. These hydroxyl radicals can bind to membrane lipids, leading to lipid peroxidation, evidenced by increased malondialdehyde (MDA or LPO) levels. Moreover, suppose hydrogen peroxide accumulates without being scavenged. In that case, it may interact with tissue chlorides in the presence of MPO to generate hypochlorous acid—another ROS that triggers pro-inflammatory responses in tissues. Besides mitigating oxidative stress, TQ exhibits an anti-inflammatory effect against HgCl_2 by reducing the NO levels that HgCl_2 stimulates through increased activity of inducible nitric oxide synthase, which raises tissue NO levels. Additionally, TQ exhibits anti-apoptotic properties in the neuronal system, which may be attributed to the inhibition of Bax (a pro-apoptotic protein) and the enhancement of Bcl-2 (an anti-apoptotic protein), resulting from a reduction in caspase-9 and caspase-3 activities. Through these mechanisms, TQ effectively modulates HgCl_2 -induced neuronal toxicities in Wistar albino rats (Fig. 14).

Future investigations should evaluate dose-response relationships, treatment duration, and potential

sex-dependent variations in TQ efficacy, supported by advanced molecular and immunohistochemical profiling of neuronal and glial injury markers. Testing in other neurotoxic models and neurons derived from human-induced pluripotent stem cells (iPSCs), along with long-term recovery studies after treatment, will help confirm the translatability of these findings to real-world clinical settings. Given its potent modulation of the Nrf-2/HO-1/Trx axis, activation of PPAR- γ , and suppression of oxidative, nitrosative, and pro-inflammatory mediators, TQ holds therapeutic potential as an adjunct neuroprotective agent against HgCl₂-induced neurodegeneration and other redox-imbalance-driven neuropathologies. However, well-controlled clinical trials are essential to determine the pharmacokinetics, bioavailability, and long-term safety of TQ in humans.

While this study provides evidence for the neuroprotective effects of TQ against HgCl₂-induced neurotoxicity, several limitations should be acknowledged. The sex-related effects, dose-related responses, and long-term effects of thymoquinone were not explored. Some mechanistic claims, including Bax/Bcl-2 modulation, were inferred from literature or docking studies rather than directly measured, and the molecular docking of thymoquinone with PPAR- γ was not validated in vitro. Additionally, more specific neuronal staining methods, such as silver or cresyl violet staining, were not employed, and markers of neuronal and glial injury or neurotransmitter alterations were not assessed. Addressing these limitations in future studies would strengthen the translational relevance of thymoquinone as a neuroprotective agent.

Conclusion

In conclusion, thymoquinone administration protects against inorganic mercury-induced neurotoxicity by improving locomotive and exploratory behaviour, decreasing cerebro-cerebellar apoptotic and pyknotic cells, through the upregulation of antioxidant and cytoprotective pathways and the downregulation of apoptosis via oxidative, inflammatory, and apoptotic trajectories. Meanwhile, further studies related to neurotransmitter synthesis, release, and metabolism should be focused on enhancing the understanding of the neuroprotective role of TQ. These results highlight the potential therapeutic value of TQ in ameliorating heavy metal-induced neurotoxicity and support further investigation into its clinical application for neuroprotection.

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Authors' contributions

All authors participated in the design, interpretation, and analysis of the data generated from the study. **SO, MO, UA, JOB, CEI, AA,** and **OO** conceptualised the study; **SO, MO, UA, JOB, CEI, AA,** and **OO**

conducted the research and preliminary data analysis. **SO, MO, UA,** and **OO s** supervised the investigation, and **SO, MO, UA, JOB, CEI, AA,** and **OO** proofread data for errors. **SO, MO, UA, JOB, CEI, AA,** and **OO** wrote and revised the manuscript.

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Data availability

The datasets used and analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The experimental protocols were approved by the Animal Welfare and Ethical Use of Laboratory Animal Committee of the University of Ibadan (UI-ACUREC) under approval number UI-ACUREC/057–1222/11).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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