jove | Neuroscience

DATE PUBLISHED March 13, 2026
DOI 10.3791/69924
URL jove.com/t/69924

Evaluating the Effect of Thymoquinone on Keap-1/Nrf-2 Signaling, Oxidative Stress, and Behavior in Rats

Dilek Kuzay Aksoy[1], **Halime Tozak Yıldız**[2]

[1]Faculty of Medicine, Department of Physiology, Kirsehir Ahi Evran University[2]Faculty of Medicine, Department of Histology and Embryology, Kirsehir Ahi Evran University

Corresponding Author : Dilek Kuzay Aksoy <dilekkuzay@ahievran.edu.tr>

Citation

Kuzay Aksoy, D., Tozak Yıldız, H. Evaluating the Effect of Thymoquinone on Keap-1/Nrf-2 Signaling, Oxidative Stress, and Behavior in Rats. *J. Vis. Exp.* (229), e69924, doi:10.3791/69924. (2026).

Abstract

Recent developments in pesticide exposure research have increased interest in antioxidant-based protective strategies. Thymoquinone (TQ), a plant-derived bioactive compound, has been reported to exert protective effects against various toxic agents. This study describes a protocol to evaluate the effects of TQ on oxidative stress parameters, the Keap-1/Nrf-2 signaling pathway, and behavioral outcomes in rats exposed to low-dose Deltamethrin (DTM). Here, 24 adult male Wistar Albino rats (250 ± 20 g) were randomly assigned to four groups (n = 6/group): control, TQ, DTM, and DTM+TQ. DTM (1.28 mg/kg) and TQ (10 mg/kg) were administered intragastrically for 30 days. Body weight was monitored throughout the study. Locomotor activity and anxiety-like behavior were assessed using the open field test on day 31, and depression-like behavior was evaluated using the forced swim test on day 32. At the end of the experimental period, plasma and brain tissues were collected for biochemical, histopathological, and molecular analyses. Oxidative stress markers, including malondialdehyde, total nitric oxide, glutathione, and sulfhydryl group levels, were measured in plasma and cerebral cortex samples. Kelch-like ECH-associated protein 1 (Keap-1) and Nuclear factor erythroid 2–related factor 2 (Nrf-2) expression levels were analyzed using Western blotting and immunohistochemistry. This protocol provides a comprehensive and reproducible approach for investigating pesticide-induced neurotoxicity and the modulatory effects of antioxidant compounds.

Introduction

Insecticides are widely used in agriculture and domestic environments to control pests; however, chronic exposure to low doses can result in environmental contamination and adverse health effects in both humans and animals. Such exposure, particularly through contaminated food and water, has been associated with an increased risk of neurological disorders and other systemic toxicities[1].

Pyrethroids have largely replaced organophosphorus insecticides due to stricter regulations on the latter and their relatively lower mammalian toxicity. DTM, a type II pyrethroid containing an α-cyano group, is among the most commonly used insecticides worldwide and is considered highly effective against insects[2]. Despite this, experimental studies have demonstrated that DTM exposure induces oxidative stress and neurotoxicity in animal models[3,4].

DTM is a highly lipophilic compound that readily crosses the blood–brain barrier and disrupts neuronal function by prolonging the opening of voltage-gated sodium channels. This effect leads to repetitive neuronal firing, increased intracellular sodium influx, and enhanced generation of reactive oxygen species, ultimately

DOI 10.3791/69924

resulting in oxidative damage, inflammation, and behavioral alterations, particularly in vulnerable brain regions such as the hippocampus and cerebral cortex[1]. Given the growing concern regarding pesticide-induced neurotoxicity, increasing attention has been directed toward antioxidant-based protective strategies derived from natural sources. TQ, a major bioactive constituent of *Nigella sativa* essential oil, has been reported to possess potent antioxidant, anti-inflammatory, and neuroprotective properties[5]. Several studies have demonstrated its beneficial effects against chemically induced oxidative stress and neurodegenerative processes[5,6,7,8,9].

One of the principal mechanisms underlying the protective effects of TQ involves modulation of the Keap-1/Nrf-2 signaling pathway. This pathway plays a central role in maintaining cellular redox homeostasis by regulating the expression of antioxidant and detoxifying enzymes. Under oxidative stress conditions, Keap-1-mediated repression of Nrf-2 is relieved, allowing Nrf-2 to translocate to the nucleus and activate antioxidant response element–dependent gene expression[9].

Because DTM exposure induces alterations at molecular, biochemical, histological, and behavioral levels, a single analytical approach is insufficient to capture the full extent of its neurotoxic effects. Therefore, this protocol integrates behavioral assessments, oxidative stress biomarker analyses, histopathological evaluation, and molecular investigation of the Keap-1/Nrf-2 pathway to provide a comprehensive and reproducible framework for studying pesticide-induced neurotoxicity and the modulatory effects of antioxidant compounds such as TQ.

Taken together, the evidence summarized above highlights the need for an integrated experimental approach to clarify the mechanisms underlying DTM-induced neurotoxicity and to evaluate potential protective interventions. By combining behavioral assessments with biochemical, histopathological, and molecular analyses of the Keap-1/Nrf-2 pathway, the present protocol enables a multidimensional evaluation of oxidative stress–related neuronal damage. The interpretation of the experimental findings within this mechanistic framework emphasizes how TQ modulates redox signaling, attenuates tissue injury, and improves behavioral outcomes in the context of subchronic DTM exposure.

Protocol

The Institutional Animal Experimentation Local Ethics Committee approved the experimental protocol under registration number 68429034/35. The total duration of the study was 32 days. Prior to the experiments, animals were acclimatized to standard laboratory conditions. All experimental procedures were conducted in strict accordance with institutional and international animal ethics guidelines. The experimental materials used in this protocol are listed in the **Table of Materials**.

NOTE: Chemical and biological wastes were disposed of in accordance with the institution's laboratory safety and waste management guidelines. Acids, bases, and organic solvents were collected in separate, labeled containers and delivered to the institutional hazardous waste unit. Biological materials and contaminated consumables were collected in biohazard containers, sterilized, and transferred to the appropriate institutional facility for final disposal.

1. Experimental design

1. Obtain 24 adult male Wistar Albino rats weighing 250 ± 20 g from the Animal Experiment Laboratory. House each rat individually in cages at 24 ± 2 °C under a 12 h light/dark cycle, with *ad libitum* access to standard rat chow and tap water.
2. Randomly divide the animals into four groups (n = 6 per group): Group 1: Control (C), Group 2: TQ, Group 3: DTM, Group 4: DTM + TQ.
3. Dissolve DTM (98% purity) in corn oil and administer at a dose of 1.28 mg/kg via intragastric gavage for 30 days.
4. Dissolve TQ at a dose of 10 mg/kg in corn oil and administer via intragastric gavage for 30 days. Prepare all drug solutions fresh daily and administer at a total volume of 1 mL/kg.
5. In the combined treatment group, administer TQ immediately after DTM. Administer corn oil alone to the control group to account for gavage-related stress.

   NOTE: The DTM dose was selected based on previous literature to induce oxidative stress without causing morbidity and corresponds to approximately 1/100 of the LD_{50}[1].

2. Measurement of body weight

1. Measure body weight on days 1, 10, 20, and 30 of the study (**Figure 1**).

3. Behavioral parameters

1. Perform all behavioral tests under standardized conditions to ensure consistency. Record both the Forced Swim Test (FST) and Open Field Test (OFT) using an overhead camera providing a full view of the testing apparatus. Maintain illumination at approximately 100–150 lux. Define immobility as the absence of active movement, except for minimal movements required to maintain posture and respiration. Clean all testing arenas with 70% ethanol between animals to prevent olfactory influences.
2. FST

DOI 10.3791/69924

1. Perform FST on day 32 to evaluate depression-like behavior. Conduct a 15-min pre-test session 24 h before the 5-min test session to habituate the animals to the water environment and minimize novelty-induced stress. Do not perform behavioral scoring during the pre-test session.
2. Individually place rats into cylindrical containers (diameter: 35 cm, height: 50 cm) containing 30 cm of water maintained at 25 ± 1 °C for 5 min.
3. Record swimming, climbing, and immobility durations using a video camera. Quantify behavioral parameters in seconds from video recordings[10].
4. Define behaviors as follows:

   Immobility: Consider the animal immobile when it remains floating without active movements, making only minimal movements necessary to keep its head above water.

   Swimming: Define swimming as active horizontal movement throughout the cylinder with coordinated movements of all four paws.

   Climbing: Define climbing as vigorous upward-directed movements of the forepaws against the cylinder wall while the animal remains in a vertical position.
5. If the animal remains completely motionless, score the behavior as immobility according to the predefined criteria.
6. Do not apply external stimulation or course correction during the test session. Allow the animal to behave freely for the entire 5-min period unless intervention is required for safety reasons (e.g., risk of drowning).

3. OFT
   1. Conduct OFT on day 31 to assess locomotor activity and anxiety-like behavior[11]. Record individual movements for 5 min in an arena measuring 90 x 35 cm, divided into 24 equal units by black lines.
   2. Quantify the following behavioral parameters from video recordings by an investigator blinded to group allocation:

      The number of crossings with all four paws (locomotor activity)

      Time spent in the center and peripheral zones (anxiety-like behavior)
   3. If an animal remains completely motionless, record the behavior as immobility and assign zero crossings for that period.
   4. Do not apply external stimulation, repositioning, or course correction during the test session.
   5. Allow the animal to behave freely for the entire 5-min period unless intervention is required for safety reasons.
   6. Clean the arena with 70% ethanol between animals to eliminate olfactory cues.

4. Assessment of oxidative stress markers

1. Perform all procedures on day 32. Deeply anesthetize rats with intramuscular xylazine (5 mg/kg) and ketamine (45 mg/kg). Confirm deep anesthesia by the absence of the pedal withdrawal reflex. Collect approximately 3–5 mL of blood via cardiac puncture into EDTA-coated tubes.
2. Complete euthanasia by exsanguination under deep anesthesia and confirm death before tissue collection. Centrifuge blood samples at 3,000 x *g* for 10–15 min at 4 °C. Carefully collect plasma without disturbing the buffy coat and store aliquots at −80 °C. Dissect the brain rapidly on ice. Separate the left cerebral hemisphere and hippocampus for biochemical analyses and snap-freeze in liquid nitrogen (−80 °C storage). Fix the right cerebral hemisphere in 10% neutral buffered formalin for histological evaluation.
3. Plasma Oxidative Stress Analyses
   1. Plasma Lipid Peroxidation (TBARS Assay)
      1. Centrifuge plasma samples (3,000 x *g*, 10 min, 4 °C). Mix 0.5 mL plasma with 1 mL of TCA–TBA–HCl reagent (15% trichloroacetic acid (TCA), 0.375% thiobarbituric acid (TBA), 0.25 N Hydrochloric acid (HCl)). Centrifuge at 1,000 x *g* for 10 min and transfer the supernatant to glass tubes. Add 50 µL of 0.02% Butylated hydroxytoluene (BHT) to prevent artificial oxidation.
      2. Heat at 100 °C for 15 min, cool to room temperature, centrifuge at 1,000 x *g* for 10 min, and measure supernatant absorbance at 532 nm[10]. Calculate MDA concentration using Beer–Lambert law

         $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$, l = 1 cm and express as nmol/mL plasma.
   2. Plasma Thiol (RSH) Levels
      1. Mix 0.5 mL of plasma with Tris-HCl buffer (100 mM, pH 8.2) containing 1% Sodium dodecyl sulfate (SDS) and 2 mM Ethylenediaminetetraacetic acid (EDTA). Incubate 5 min at 25 °C and centrifuge (3,000 x *g*, 10 min, 4 °C). Use the supernatant for analysis.
      2. Add 0.3 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and incubate at 37 °C for 15 min. Measure absorbance at 412 nm[10]. Calculate RSH

DOI 10.3791/69924

concentrations using ε = 13,600 M^{-1} cm^{-1} and express as µmol/L plasma.

3. Plasma NOx Levels (Griess Reaction)
    1. Deproteinize plasma with 0.3 M Sodium hydroxide (NaOH) and 5% zinc sulfate ($ZnSO_4$). Centrifuge at 20,000 x *g* for 5 min and collect the supernatant.
    2. Prepare nitrate stock solutions (100, 10, and 1 mmol/L) and generate a standard curve (1–100 µmol/L). Load 100 µL of supernatant (duplicate wells) into a microplate.
    3. Add 100 µL of vanadium (III) chloride (VCl_3) followed by 50 µL of sulfanilamide and 50 µL of N-(1-naphthyl) ethylenediamine. Incubate 30–45 min at room temperature and measure absorbance at 540 nm[10]. Determine NOx concentrations from the standard curve and express as µmol/L plasma.
4. Tissue oxidative stress analyses
    1. Use approximately 100 mg of left cerebral hemisphere tissue for all biochemical analyses.
    2. Tissue lipid peroxidation (TBARS Assay)
        1. Homogenize tissue (1:10 w/v) in ice-cold 10% TCA. Add an equal volume of 0.67% TBA and heat at 100 °C for 15 min. Cool and centrifuge (3,000 x *g*, 10 min, 4 °C)[10]. Measure supernatant absorbance at 535 nm. Calculate MDA using ε = 1.56 × 10^5 M^{-1} cm^{-1} and express as nmol/g wet tissue.
    3. Tissue glutathione (GSH) levels
        1. Homogenize tissue in 10% TCA (1:10 w/v) and centrifuge (3,000 x *g*, 10 min, 4 °C). Mix 0.5 mL of supernatant with 2 mL of DTNB solution (0.4 mg/mL in 1% sodium citrate)[10]. Measure absorbance at 412 nm immediately. Calculate GSH using ε = 13,600 M^{-1} cm^{-1} and express as µmol/g wet tissue.
    4. Tissue NOx levels (Griess Reaction)
        1. Homogenize tissue in phosphate-buffered saline (PBS) (1:5 w/v) and centrifuge (2,000 x *g*, 5 min). Deproteinize supernatant with 0.3 M NaOH and 5% $ZnSO_4$. Centrifuge (3,000 x *g*, 20 min) and collect the clear supernatant.
        2. Prepare nitrate standards (1–100 µmol/L). Load 100 µL of supernatant in duplicate wells. Add VCl_3 and Griess reagents as described above. Incubate 45 min at 37 °C and measure absorbance at 540 nm[10]. Determine concentrations from the standard curve and express as µmol/g tissue.

CAUTION: Procedures involving hazardous chemicals, including TCA, HCl, NaOH, and organic solvents, were performed in a certified chemical fume hood. Appropriate personal protective equipment (PPE), such as laboratory coats, chemical-resistant gloves, protective eyewear, and, when necessary, face shields, was used throughout all experiments. All chemicals were handled according to institutional safety guidelines and relevant material safety data sheets (MSDS).

5. Determination of Keap-1 and Nrf-2 Levels by Western blot analysis

1. Cut hippocampal tissue into 50 mg pieces and place into homogenization tubes containing 500 µL of RIPA buffer supplemented with protease inhibitor cocktail.
2. Add an appropriate amount of magnetic beads and homogenize at 1,000 x *g* for two cycles. Centrifuge at 14,000 x *g* for 10 min at 4 °C.
3. Transfer the supernatant to clean microcentrifuge tubes and centrifuge again under the same conditions. Keep samples on ice and determine protein concentrations.
4. Measure protein concentrations using a fluorometer with the Protein BR Assay Kit, following the manufacturer's instructions.
5. Prepare 10% SDS–PAGE gels for electrophoresis. Adjust protein concentration to 30 µg/µL.
6. Add 4x Laemmli buffer at a 3:1 ratio and supplement with 10% β-mercaptoethanol. Heat samples at 95 °C for 5 min, then briefly centrifuge.
7. Load 5 µL of protein marker and 30 µg protein per lane. Run gels at 110 V for 60–90 min.
8. Activate PVDF membranes in 100% methanol for 3–5 min, then equilibrate in 1× transfer buffer for 10 min at room temperature before transfer.
9. Wet filter papers and sponges with transfer buffer and assemble the transfer sandwich carefully, ensuring complete removal of air bubbles. Perform protein transfer using a wet transfer system at 100 V for 90 min at 4 °C (constant voltage).
10. Block membranes in 3% Bovine Serum Albumin (BSA) for 1 h at room temperature on an orbital shaker. Incubate membranes overnight at 4 °C with primary antibodies diluted in 3% BSA (Keap-1 and Nrf-2, 1:3,000; β-actin, 1:1,000).
11. Wash membranes 5x for 5 min with 1 × Tris-buffered saline with 0.1% Tween 20 detergent (TBS-T). Incubate with secondary antibody (1:15,000) for 1 h at room temperature in the dark.

DOI 10.3791/69924

12. Wash membranes as described and incubate with an enhanced chemiluminescence (ECL) substrate (e.g., luminol-based HRP substrate) according to the manufacturer's instructions (mixing equal volumes of reagent A and B immediately before use). Apply sufficient substrate to completely cover the membrane surface (approximately 1 mL per membrane) and incubate for 1–2 min at room temperature.
13. Detect chemiluminescent signals using a digital imaging system equipped for chemiluminescence detection (no specific excitation wavelength required, as light emission results from HRP–luminol reaction). Acquire images using automatic exposure settings while ensuring non-saturated bands. Maintain identical exposure parameters for all samples within the same blot.
14. Perform densitometric analysis using Image analysis software under identical ROI selection, background subtraction, and normalization parameters. Normalize Keap-1 and Nrf-2 band intensities to the corresponding housekeeping protein β-actin. Export normalized values as .csv files for statistical analysis.

6. Histopathological analysis

1. Fix the right cerebral hemisphere in 10% neutral formalin for 72 h. Dehydrate through a graded ethanol series (70%–100%), embed in paraffin, and section at 5 µm.

   Obtain coronal brain sections at the hippocampal and entorhinal cortex levels according to the stereotaxic coordinates defined in Paxinos & Watson, The Rat Brain in Stereotaxic Coordinates (Academic Press). Use the interactive rat brain atlas viewer (https://labs.gaidi.ca/rat-brain-atlas to verify and refine anatomical localization[11].
2. Prepare four consecutive coronal sections (5 µm thickness) from paraffin-embedded brain tissue using standard histological procedures. Stain the sections with hematoxylin and eosin (H&E) according to conventional protocols[12]. Examine all slides under a light microscope at 200× magnification. Ensure that the histological evaluation is performed by a blinded investigator to minimize observer bias[13].

   NOTE: This procedure ensured consistent anatomical comparison across groups.
3. Semiquantitatively assess neuronal loss, cellular edema, nuclear pyknosis, inflammatory infiltration, and vascular congestion in 10 random fields per section.
4. Calculate mean scores for each parameter per group. Scoring criteria were as follows: 0 (none, <5%), 1 (mild, <33%), 2 (moderate, 33–66%), and 3 (severe, >66%; **Table 1).**

7. Immunohistochemical analysis of Nrf-2 and Keap-1 reactivity in the hippocampus and entorhinal cortex

1. Prepare 5 µm thick sections from the hippocampus and entorhinal cortex and mount them on glass slides.
2. Deparaffinize and rehydrate the sections, then wash in phosphate-buffered saline (PBS) at room temperature.
3. Incubate sections with 3% hydrogen peroxide for 5 min to block endogenous peroxidase activity. Wash sections with PBS
4. Perform antigen retrieval by heating the slides in sodium citrate buffer (pH 6.0) for 10–15 min. Allow the slides to cool for 30 min, then wash with PBS.
5. Incubate sections again with 3% hydrogen peroxide for 15 min to ensure complete peroxidase inhibition. Wash sections with PBS.
6. Apply a blocking solution for 10 min to reduce nonspecific binding. Incubate the sections overnight at 4 °C with primary antibodies against Nrf-2 (1:100 dilution) and Keap-1 (1:200 dilution). Wash the sections with PBS after incubation.
7. Incubate sections with a suitable secondary antibody for 30 min at room temperature. Wash sections with PBS.
8. Apply streptavidin–peroxidase to the sections and incubate for 20 min at room temperature in a humidified chamber (maintain 95–100% relative humidity using a sealed incubation box containing moistened filter paper). Wash the sections with PBS following incubation.
9. Apply DAB chromogen and monitor color development under a light microscope at 100× magnification. Stop the reaction by rinsing the slides with tap water.
10. Counterstain the sections with hematoxylin diluted 1:9 for 5 min at room temperature. Rinse thoroughly with PBS and distilled water. Mount the sections using a permanent mounting medium.
11. Examine the stained sections under a light microscope. Capture photomicrographs at ×400 magnification from 16 representative fields per section.
12. Quantify Nrf-2 and Keap-1 immunoreactivity using image analysis software. Record and analyze the quantified data for further statistical evaluation.
13. For immunohistochemical analysis, open the images in ImageJ and calibrate using the Set Scale tool. Convert all images to 8-bit grayscale to standardize intensity measurements. Manually define regions of interest (ROIs) for positively stained areas and adjacent background regions and save them in the ROI Manager.

DOI 10.3791/69924

14. Quantify staining intensity using consistent H-DAB color deconvolution, followed by identical thresholding parameters across all samples. Measure mean gray value, optical density, and percentage of positive area.
15. For cell-based analysis, segment thresholded images using the Watershed tool, and quantify positive cells using Analyze Particles. All data were exported as .csv files for statistical analysis.

8. Statistical analysis

1. Perform statistical analyses using SPSS version 29.0. Enter raw data in the Data View tab, with each row representing an individual animal and variables defined in the Variable View tab; the experimental group was coded as a categorical variable (Control, TQ, DTM, and DTM+TQ).
2. Assess data normality using the Shapiro–Wilk test by selecting **Analyze > Descriptive Statistics > Explore**, assigning outcome variables to the Dependent List and Group to the Factor List, and enabling Normality plots with tests. For normally distributed variables, conduct group comparisons using one-way analysis of variance (ANOVA) via **Analyze > Compare Means > One-Way ANOVA**, with descriptive statistics and Levene's test for homogeneity of variances enabled under Options.
3. When a significant main effect was detected, apply Tukey's post hoc test through the Post Hoc menu to identify pairwise group differences. Use Levene's test results to confirm variance homogeneity ($p > 0.05$).
4. For histopathological scoring data that did not meet parametric assumptions, perform nonparametric analysis using the Kruskal–Wallis test by selecting **Analyze > Nonparametric Tests > Legacy Dialogs > K Independent Samples**, followed by Dunn's post hoc test with Bonferroni correction using the Independent Samples nonparametric test module.
5. Export statistical outputs and summary tables as .xls or .csv files, results were expressed as mean ± standard deviation (SD), and a p-value < 0.05 was considered statistically significant.

Results

Body weight measurements

Body weight was recorded on days 1, 10, 20, and 30. Rats in the C and TQ groups showed a progressive increase in body weight over the experimental period, with no significant difference between these groups on day 30. In contrast, rats exposed to DTM exhibited a significant reduction in body weight compared with controls. Co-administration of TQ attenuated DTM-associated weight loss. No mortality was observed during the study period **(Figure 1).**

Behavioral assessments

Locomotor activity

Locomotor activity was assessed by the number of grid crossings in the open-field test. The C and TQ groups displayed comparable locomotor activity. DTM exposure significantly reduced the number of crossings, whereas TQ co-administration partially increased locomotor activity compared with the DTM group **(Figure 2).**

OFT

Exploratory behavior was evaluated by measuring time spent in the center and periphery of the arena. The C and TQ groups showed similar center–periphery distributions. DTM exposure resulted in decreased time spent in the center and increased time in the periphery. The DTM+TQ group exhibited intermediate values between control and DTM-treated animals **(Figure 3).**

FST

Immobility, swimming, and climbing times were recorded to assess depression-like behavior. The TQ group did not differ significantly from controls. DTM exposure significantly increased immobility time and reduced swimming and climbing durations. In the DTM+TQ group, immobility time was reduced and swimming time was increased compared with the DTM group, while climbing time remained unchanged **(Figure 4).**

Oxidative stress markers

Cerebral cortex

Cortical levels of MDA, NOx, and GSH were measured. TQ treatment alone significantly increased GSH levels compared with controls. DTM exposure significantly increased MDA and NOx levels and decreased GSH levels. In the DTM+TQ group, these parameters were partially restored toward control values **(Table 2).**

Plasma

In plasma samples, TQ treatment increased RSH levels and decreased NOx levels, while MDA levels remained unchanged. DTM exposure significantly increased MDA and NOx levels and decreased RSH levels. Co-administration of TQ partially reversed these alterations **(Table 3).**

Western blot analysis

Western blot analysis revealed decreased Keap-1 and increased Nrf-2 protein expression in the TQ group compared with controls. DTM exposure significantly increased Keap-1 and decreased Nrf-2 expression. In the DTM+TQ group, both protein levels were modulated toward control values **(Figure 5).**

Histopathology

DOI 10.3791/69924

Hematoxylin and eosin–stained cortical sections from the C and TQ groups showed preserved neuronal morphology and tissue architecture. In contrast, DTM-treated rats exhibited neuronal shrinkage, pyknotic nuclei, cytoplasmic and pericellular edema, vacuolization, inflammatory cell infiltration, microglial activation, and vascular congestion. These pathological features were reduced in the DTM+TQ group, although mild alterations persisted **(Figure 6).**

Histopathological scoring

Semi-quantitative scoring demonstrated significantly increased neuronal damage, edema/pyknosis, inflammation, and vascular congestion in the DTM group compared with controls. TQ co-administration significantly reduced these scores, although values did not fully return to control levels. Inflammatory cell infiltration scores in the DTM+TQ group were comparable to those of the control group **(Table 4).**

Immunohistochemistry

In the C and TQ groups, Nrf-2 immunoreactivity was predominantly cytoplasmic, and Keap-1 staining was weak. DTM exposure markedly decreased Nrf-2 immunoreactivity and increased Keap-1 staining intensity. The DTM+TQ group exhibited increased Nrf-2 immunoreactivity, including nuclear localization, and reduced Keap-1 staining compared with the DTM group **(Figure 7).**

Quantitative analysis of immunostaining

Quantitative analysis confirmed a significant reduction in Nrf-2 and an increase in Keap-1 immunoreactivity in DTM-treated rats relative to controls. TQ co-administration significantly improved both parameters toward control levels, although complete normalization was not observed **(Figure 8).**

The representative results presented here demonstrate the successful application and internal consistency of the behavioral, biochemical, molecular, and histopathological techniques described in the protocol. The open-field and forced swim tests reliably detected DTM-induced alterations in locomotor and depression-like behaviors, confirming the sensitivity of these paradigms to neurotoxic and antioxidant interventions. Spectrophotometric assays (TBARS, thiol, GSH, and Griess-based NOx measurements) generated reproducible absorbance values that translated into quantifiable oxidative stress markers using the Beer–Lambert law, illustrating the robustness of colorimetric detection when appropriate blanks and calibration curves are applied. Western blot analysis yielded clearly distinguishable bands for Keap-1 and Nrf-2, and normalization to β-actin enabled semi-quantitative comparison of protein expression across groups, highlighting the importance of consistent exposure and densitometric parameters. Similarly, immunohistochemical staining demonstrated spatial distribution and intensity differences in Nrf-2 and Keap-1 reactivity, validating the utility of standardized antigen retrieval, thresholding, and ROI-based quantification. Together, these findings confirm that the described methodological workflow is suitable for detecting oxidative stress–related molecular and behavioral changes and emphasize the necessity of maintaining identical acquisition, normalization, and analysis parameters to ensure reproducibility.

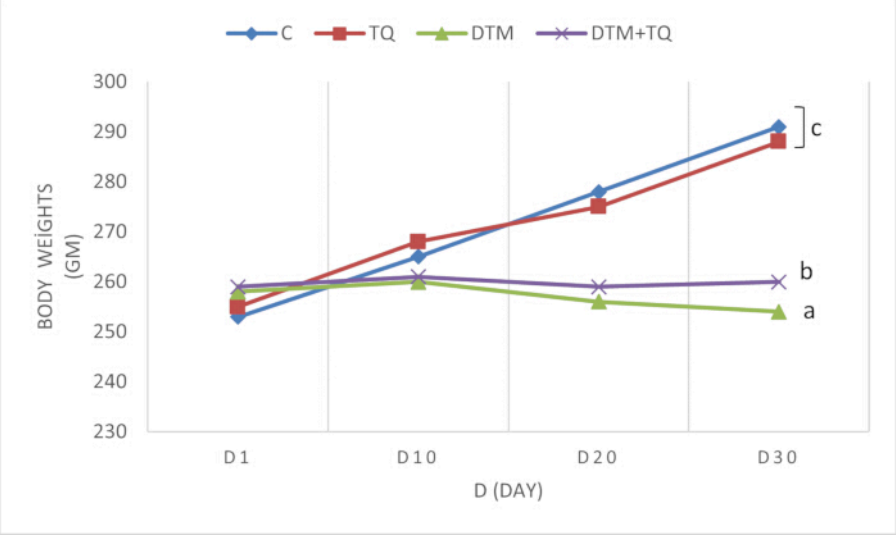

Figure 1: Representative body weight measurements on days 1, 10, 20, and 30. Statistical comparison of body weight results measured on day 30. Data represent mean ± SD (n = 6). Rats in the C and TQ groups show a normal increase in body weight across the study period, whereas DTM exposure results in a marked reduction by day 30. Co-treatment with TQ partially restores body weight in the DTM+TQ group. a: $p < 0.001$ vs. C and TQ; b: $p < 0.001$ vs. DTM; c: $p = 0.3$, not significant. Please click here to view a larger version of this figure.

DOI 10.3791/69924

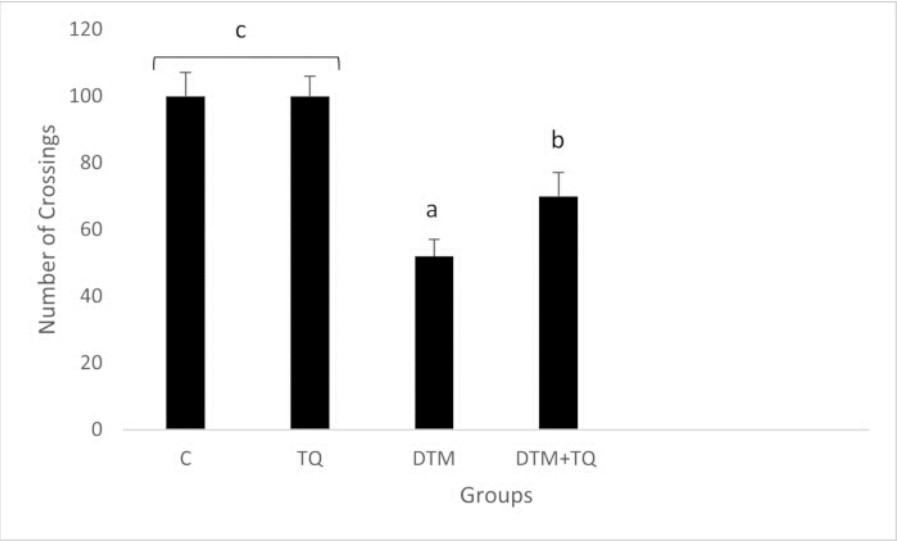

Figure 2: Representative locomotor activity quantified as the number of crossings in the OFT. Mean ± SD (n = 6). C and TQ groups exhibit baseline locomotor activity. DTM exposure reduces locomotion, while TQ co-administration partially restores crossing frequency. a: $p < 0.001$ vs. C and TQ; b: $p < 0.001$ vs. DTM; c: $p = 0.99$, not significant. Please click here to view a larger version of this figure.

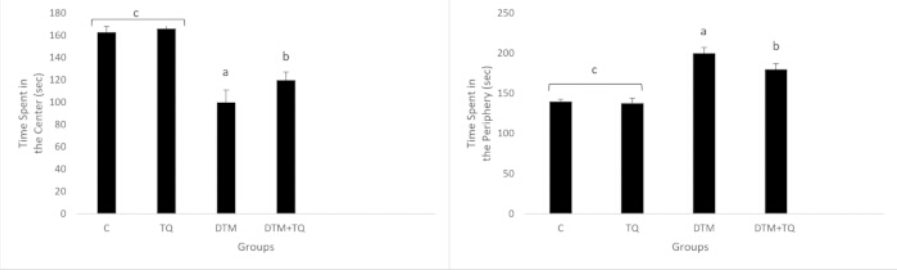

Figure 3: Effects of DTM and TQ on anxiety-like behavior in the open field test (OFT). (A) Time spent in the center zone (s). **(B)** Time spent in the periphery (s). Mean ± SD (n = 6) Normal exploratory behavior is observed in the C and TQ groups. DTM exposure decreases center time and increases peripheral time, indicating anxiety-like behavior. TQ co-treatment increases center exploration and reduces peripheral preference relative to the DTM group. a: $p < 0.001$ vs. C and TQ; b: $p < 0.001$ vs. DTM; c: $p = 0.9$, not significant. Please click here to view a larger version of this figure.

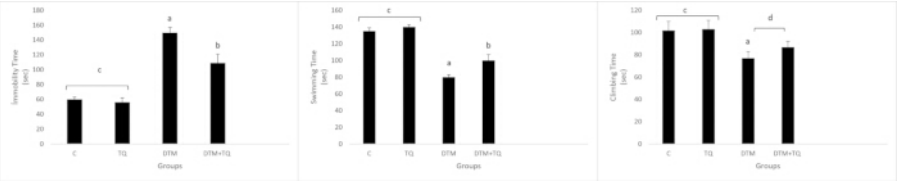

Figure 4: Effects of DTM and TQ on depressive-like behavior in the forced swim test (FST). (A) Immobility time (s). **(B)** Swimming time (s). **(C)** Climbing time (s). Mean ± SD (n = 6). DTM exposure increases immobility and reduces climbing and swimming behaviors, indicating depressive-like behavior. TQ co-treatment reduces immobility toward control levels and partially restores swimming behavior, whereas climbing time shows minimal recovery. a: $p < 0.001$ vs. C and TQ; b: $p < 0.001$ vs. DTM; d: not significant (climbing $p = 0.14$); c: not significant (immobility $p = 0.8$; climbing $p = 0.99$; swimming $p = 0.25$). Please click here to view a larger version of this figure.

DOI 10.3791/69924

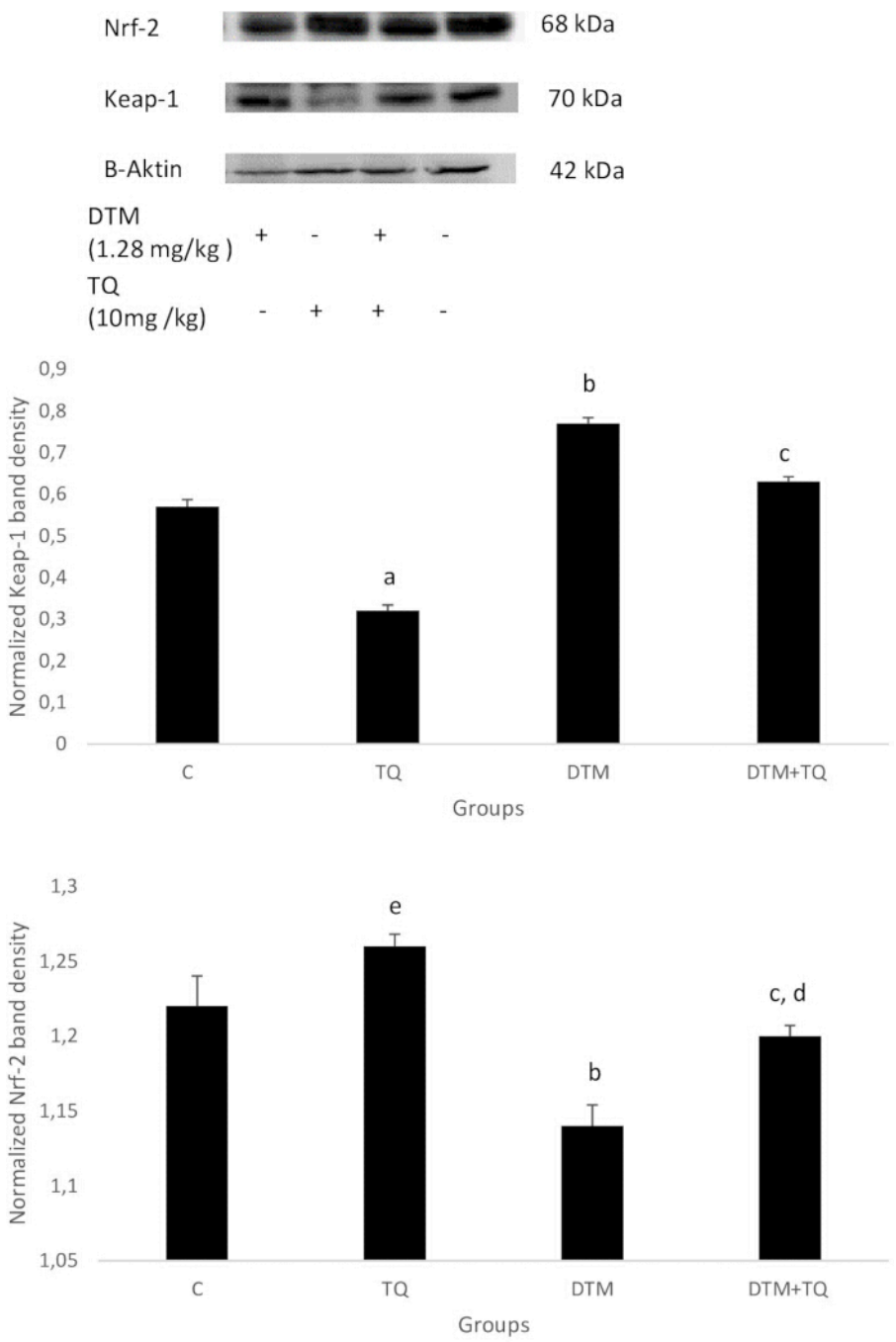

Figure 5: Effects of DTM and TQ on Keap-1 and Nrf-2 protein expression in hippocampal tissue. (A) Representative Western blot images showing Keap-1 and Nrf-2 protein expression levels. DTM exposure increases Keap-1 and decreases Nrf-2 expression, whereas TQ co-treatment partially normalizes these alterations. **(B)** Normalized Keap-1 band density. **(C)** Normalized Nrf-2 band density. β-actin was used as a loading control. Mean ± SD (n = 6). DTM significantly elevates Keap-1 and suppresses Nrf-2 expression compared to controls, while TQ co-treatment reduces Keap-1 levels and restores Nrf-2 expression toward control values. a, b: $p < 0.001$ vs. C; c: $p < 0.001$ vs. DTM; e: $p = 0.002$ vs. C; d: $p = 0.18$, not significant vs. C. The raw Western blots are provided in **Supplementary File 1**. Please click here to view a larger version of this figure.

DOI 10.3791/69924

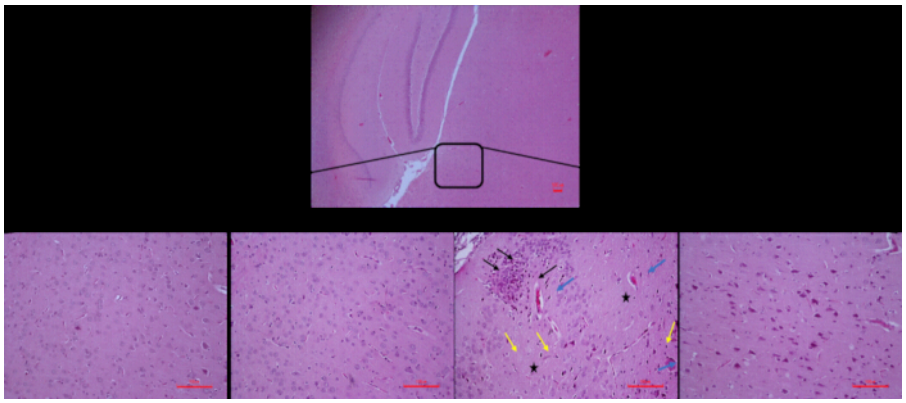

Figure 6: Representative H&E-stained coronal sections of the hippocampus and entorhinal cortex (scale bar: 100 µm). C and TQ groups display intact neuronal architecture. DTM exposure induces characteristic neurotoxic changes: neuronal loss (black stars), edema, nuclear pyknosis (yellow arrows), inflammatory cell infiltration/microglial accumulation (black arrows), and vascular congestion (blue arrowheads). DTM+TQ shows partial restoration with reduced inflammatory infiltration and preserved neuronal morphology. Please click here to view a larger version of this figure.

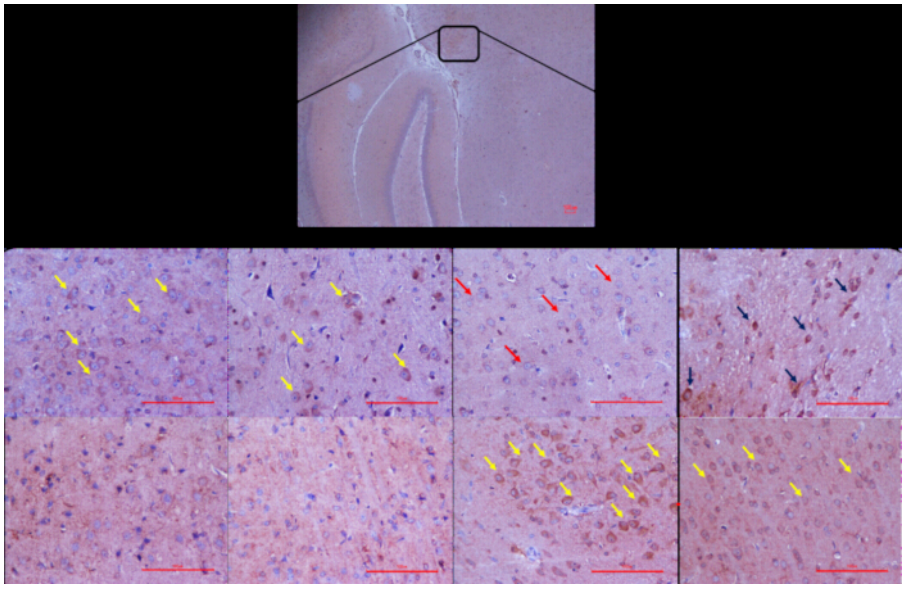

Figure 7: Representative immunohistochemical staining for Nrf-2 and Keap-1 at the hippocampal and entorhinal levels (scale bar: 100 µm; magnification 400x). C and TQ groups exhibit cytoplasmic Nrf-2 staining and low Keap-1 reactivity. DTM reduces Nrf-2 (red arrows) and increases Keap-1 staining. TQ restores Nrf-2 localization (cytoplasmic + nuclear) and reduces Keap-1 intensity. Please click here to view a larger version of this figure.

DOI 10.3791/69924

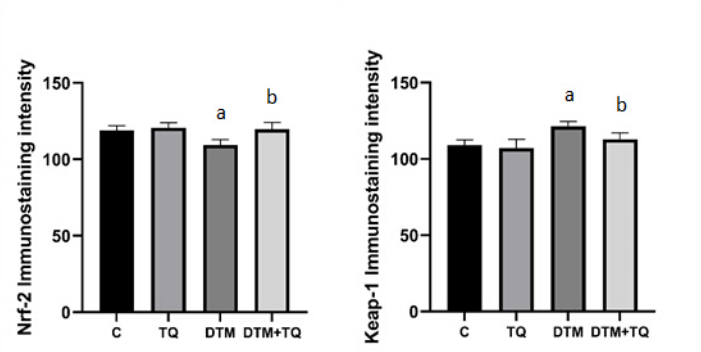

Figure 8: Quantitative immunostaining intensity of Nrf-2 and Keap-1. Mean ± SD (n = 6). DTM significantly reduces Nrf-2 and increases Keap-1 staining. TQ co-treatment significantly reverses both markers. a: $p < 0.001$ vs. C and TQ; b: Nrf-2 $p < 0.001$; Keap-1 $p = 0.007$ vs. DTM. Please click here to view a larger version of this figure.

Parameter	Score 0	Score 1 (Mild)	Score 2 (Moderate)	Score 3 (Severe)
Neuronal Loss	Normal neuronal density, no loss	Mild rarefaction, few neurons lost	Marked reduction in neuronal density, focal loss	Extensive neuronal loss with large empty areas or necrosis
Cellular Edema & Nuclear Pyknosis	None	Occasional pyknotic cells, mild edema	Frequent pyknotic cells, moderate edema	Widespread pyknotic neurons, severe edema and necrotic appearance
Inflammatory Cell Infiltration	None	Scattered inflammatory cells	Moderate perivascular/ parenchymal infiltration	Diffuse infiltration with high inflammatory cell density
Vascular Congestion	Normal vascular morphology	Slight capillary dilatation	Noticeable vascular engorgement	Severe congestion with perivascular edema

Table 1: Semi-quantitative histopathological scoring criteria (0–3). Representative scoring system used to assess neuronal loss, edema/pyknosis, inflammatory infiltration, and vascular congestion.

DOI 10.3791/69924

Cerebral Cortex			
	MDA Levels	NOx Levels	GSH Levels
	(nmol/g)	(µmol/g)	(nmol/g)
C	3,98±0,22	0,26±0,007	4,9±0,02
TQ	3,91±0,19	0,24±0,005	5,3±0,02 c
DTM	6,88±0,23 a	0,36±0,005 a	3,8±0,03 a
DTM+TQ	5,39±0,23 b	0,30±0,007 b	4,4±0,02 b

Table 2: Cerebral cortex MDA, NOx, and GSH levels. Mean ± SD (n = 6). DTM increases MDA and NOx while reducing GSH; TQ co-treatment partially reverses these effects. a: $p < 0.001$ vs. C and TQ; b: $p < 0.001$ vs. DTM; c: $p < 0.001$ vs. C.

Plasma			
	MDA Levels	NOx Levels	RSH Levels
	(nmol/ml)	(µmol/ml)	(nmol/ml)
C	0,43±0,049	7,38±0,2	154,1±1,46
TQ	0,41±0,031	6,85±0,16 c	159,5±1,81 c
DTM	0,74±0,04 a	11,9±0,28 a	136,53±1,86 a
DTM+TQ	0,59±0,038 b	9,5±0,36 b	147,4±1,63 b

Table 3: Plasma MDA, NOx, and GSH/RSH levels. Mean ± SD (n = 6). DTM increases MDA and NOx while reducing RSH; TQ co-treatment normalizes oxidative stress markers. a: $p < 0.001$ vs. C and TQ; b: $p < 0.001$ vs. DTM; c: $p < 0.001$ vs. C.

DOI 10.3791/69924

Parameter	C	TQ	DTM	DTM+QT
Neuronal Loss	0.17[0.0-1.0] [a]	0.50[0.0-1.0][a]	2.50[2.0-3.0] [c]	1.00[1.0-2.0] [b]
Cellular Edema&	0.17[0.0-1.0] [a]	0.17[0.0-1.0][a]	2.17[2.0-3.0] [c]	0.50[0.0-1.0] [b]
Nuclear Pyknosis				
Inflammatory Cell Infiltration	0.33[0.0-1.0] [a]x	0.50[0.0-1.0] [a]	2.33[2.0-3.0][c]	0.33[0.0-1.0] [a]
Vascular Congestion	0.33[0.0-1.0] [a]	0.67[0.0-1.0] [a]	1.83[1.0-3.0] [c]	1.33[1.0-2.0] [b]

Table 4: Histopathological scoring of the hippocampus and entorhinal cortex. Semi-quantitative histopathological scores presented as median (min–max) for neuronal loss, cellular edema/nuclear pyknosis, inflammatory cell infiltration, and vascular congestion. Different superscript letters indicate statistically significant differences between groups. Mean ± SD (n = 6). *b: p < 0.05 vs. DTM; c: p < 0.001 vs. C and TQ.*

Supplementary File 1: Raw Western blots. Please click here to download this file.

Discussion

This study demonstrates that subchronic oral exposure to DTM induces consistent behavioral, biochemical, molecular, and histopathological alterations in rats and that TQ effectively mitigates these effects when administered concomitantly. By integrating standardized behavioral tests with oxidative stress profiling, pathway-level analysis of Keap-1/Nrf-2 signaling, and histopathological evaluation, the protocol provides a robust and reproducible framework for assessing pesticide-induced neurotoxicity and antioxidant-based neuroprotection.

The selected DTM dose (1.28 mg/kg/day, oral gavage, 30 days) was based on established subchronic exposure models and produced measurable neurobehavioral and biochemical effects without inducing mortality or acute systemic toxicity[14]. Consistent with previous studies using similar dosing regimens and exposure routes, DTM-treated rats exhibited body-weight loss, reduced locomotor activity, increased immobility in the FST, and decreased center exploration in the OFT[1,15,16,17,18,19,20,21]. Variations reported in the literature, including antidepressant-like responses following acute or intraperitoneal administration[22], underscore the importance of exposure route, dose, and duration when interpreting behavioral outcomes. The present findings align closely with oral subchronic models, supporting the validity of the experimental design.

Co-administration of TQ (10 mg/kg/day, oral) significantly attenuated DTM-induced behavioral impairments, partially restoring locomotor activity, reducing immobility, and improving exploratory behavior. Although TQ has not previously been examined in a DTM neurotoxicity model, similar behavioral improvements have been reported in other neurotoxicant paradigms using comparable dosing strategies[23,24,25,26]. These results indicate that the behavioral assays employed in this protocol are sensitive to detecting both neurotoxic effects and therapeutic modulation.

At the biochemical and molecular levels, DTM exposure increased lipid peroxidation and nitric oxide metabolites while reducing glutathione/thiol levels in brain tissue and plasma, accompanied by upregulation of Keap-1 and suppression of Nrf-2 expression. These findings are consistent with studies applying analogous oxidative stress assays and protein analyses under comparable exposure conditions[27,28,29,30,31]. Reports of increased Nrf-2 expression under acute or high-dose conditions[16,32,33] likely reflect compensatory responses related to methodological differences. In the present protocol, TQ treatment effectively reversed DTM-induced oxidative imbalance and normalized Keap-1/Nrf-2 signaling, in agreement with studies demonstrating antioxidant and Nrf-2–modulating effects of TQ in pesticide and toxin exposure models[8,34,35,36]. Histopathological analysis further confirmed neuronal degeneration, inflammatory infiltration, and vascular alterations following DTM exposure, consistent with previous reports using similar oral dosing paradigms[20,28]. TQ co-treatment markedly reduced these pathological features, supporting the concordance between biochemical recovery, molecular pathway modulation, and structural preservation observed in this integrated workflow.

Several procedural steps are critical for reproducibility and data quality. Accurate daily dosing, consistent gavage technique, and strict control of environmental conditions during behavioral testing are essential to minimize variability. Rapid tissue collection, standardized processing, and careful handling of redox-sensitive

samples are necessary to preserve biochemical integrity. Blinded histopathological and immunohistochemical analyses further strengthen internal validity. Despite these controls, limitations inherent to animal behavioral testing, semi-quantitative scoring, and Western blot normalization should be considered when interpreting results.

Overall, this protocol offers a comprehensive and adaptable approach for investigating pesticide-induced neurotoxicity and antioxidant-based interventions. Its modular design allows extension to other toxicants, signaling pathways, tissues, and behavioral paradigms, providing a practical framework for mechanistic and therapeutic studies. The present findings support TQ as a promising neuroprotective agent and highlight the Keap-1/Nrf-2 axis as a key target in DTM-induced oxidative neurotoxicity.

A limitation of the present study is that endoplasmic reticulum stress and apoptosis-related markers were not evaluated; future studies incorporating these pathways may provide additional mechanistic insight into thymoquinone-mediated neuroprotection.

Disclosures

The authors do not have any conflicts of interest or competing financial interests.

Acknowledgments

This research received no external funding.

References

1. Ogut, E. et al. Protective effects of syringic acid on neurobehavioral deficits and hippocampal tissue damages induced by sub-chronic deltamethrin exposure. *Neurotoxicol. Teratol.* **76**, 106839 (2019).
2. Souza, M. F. et al. Motor, memory, and anxiety-like behavioral impairments associated with brain-derived neurotrophic factor and dopaminergic imbalance after inhalational exposure to deltamethrin. *Brain Res Bull.* **181**, 55-64 (2022).
3. Chandra, N., Jain, N. K., Sondhia, S., Srivastava, A. B. Deltamethrin induced toxicity and ameliorative effect of alpha-tocopherol in broilers. *Bull Environ Contam Toxicol.* **90** (6),673-678 (2013).
4. Sharma, P., Jan, M., Singh, R. Deltamethrin toxicity. *Int J Biol Stud Res.* **2** (2), 91-107 (2013).
5. Abdulbaki, H., Al-Deeb, M. A. Neuroprotective effects of ferulic acid and thymoquinone against deltamethrin-induced neurotoxicity in Drosophila melanogaster. *Adv Life Sci.* **10** (2), 289-297 (2023).
6. Ince, S., Kucukkurt, I., Demirel, H. H., Turkmen, R., Sever, E. Thymoquinone attenuates cypermethrin induced oxidative stress in Swiss albino mice. *Pestic Biochem Physiol.* **104**, 229-235 (2012).
7. Hafez, E., El-Atrash, A., Massoud, A., Attia, M. The impact of the organophosphate insecticide "Malathion" on rat and the possible ameliorating role of "Thymoquinone". *Egypt J Exp Biol (Zool.).* **13** (2), 309-318 (2017).
8. Imam, A. L. et al. Orally administered Thymoquinone mitigates cypermethrin-induced dentate gyrus oxidative stress, preventing GABAergic interneuron degeneration and memory impairment in rats via the Nrf2/ARE pathway. *Mol Neurobiol.* **61**, 20 (2024).
9. Dong, J. et al. Thymoquinone Prevents Dopaminergic Neurodegeneration by Attenuating Oxidative Stress Via the Nrf2/ARE Pathway. *Front Pharmacol.* **14** (11), 615598 (2021).
10. Kuzay, D., Dileköz, E., Özer, Ç. Effects of thymoquinone in a rat model of reserpine-induced depression. *Braz J Pharm Sci.* **58**, e19847 (2022).
11. Paxinos, G., Watson, C. *The Rat Brain in Stereotaxic Coordinates.* 7th ed. Academic Press (2013).
12. Bancroft, J. D., Gamble, M. *Theory and Practice of Histological Techniques.* 7th ed. Churchill Livingstone (2013).
13. Klopfleisch, R. Multiparametric and semiquantitative scoring systems for the evaluation of mouse model histopathology. *BMC Vet Res.* **9**, 123 (2013).
14. Brocardo, P. S. et al. Anxiety- and depression-like behaviors are accompanied by an increase in oxidative stress in a rat model of fetal alcohol spectrum disorders: Protective effects of voluntary physical exercise. *Neuropharmacology.* **62**, 1607-1618 (2012).
15. Arora, D. et al. Evaluation and physiological correlation of plasma proteomic fingerprints for deltamethrin-induced hepatotoxicity in Wistar rats. *Life Sci.* **160**, 72-83 (2016).
16. Zaki, S. M., Algaleel, W. A. A., Imam, R. A., Soliman, G. A., Ghoneim, F. M. Nano curcumin versus curcumin in amelioration of deltamethrin induced hippocampal damage. *Histochem Cell Biol.* **154**, 157-175 (2020).
17. Zhang, J. et al. Exposure to deltamethrin in adolescent mice induced thyroid dysfunction and behavioral disorders. *Chemosphere.* **241**, 125118 (2020).
18. Ahmed, W. M. S., Abdel-Azeem, N. M., Ibrahim, M. A., Helmy, N. A., Radi, A. M. Neuromodulatory effect of cinnamon oil on behavioural disturbance, CYP1A1, İnos transcripts and neurochemical alterations induced by deltamethrin in rat brain. *Ecotoxicol Environ Saf.* **209**, 111820 (2021).
19. Khalifa, A. G. et al. Deltamethrin and Its Nanoformulations Induce Behavioral Alteration and Toxicity in Rat Brain through Oxidative Stress and JAK2/STAT3 Signaling Pathway. *Toxics.* **10**, 303 (2022).
20. Khalil, H. M. A. et al. Selenium nanoparticles impart robust neuroprotection against deltamethrin-induced neurotoxicity in male rats by reversing behavioral alterations, oxidative damage, apoptosis, and neuronal loss. *Neurotoxicology.* **91**, 329-339 (2022).
21. Lazarinia, C. A., Florioa, J. C., Lemonicab, I. P., Bernardi, M. M. Effects of prenatal exposure to deltamethrin on forced swimming behavior, motor activity, and striatal dopamine levels in male and female rats. *Neurotoxicol. Teratol.* **23**, 665-673 (2001).
22. Takasaki, I. et al. Type II pyrethroid deltamethrin produces antidepressant-like effects in mice. *Behav Brain Res.* **257**, 182-188 (2013).
23. Ramachandran, S., Thangarajan, S. A novel therapeutic application of solid lipid nanoparticles encapsulated thymoquinone (TQ-SLNs) on 3-nitropropionic acid induced Huntington's disease-like symptoms in wistar rats. *Chem Biol Interact.* **256**, 25-36 (2016).
24. Ramachandran, S., Thangarajan, S. Thymoquinone loaded solid lipid nanoparticles counteracts 3-Nitropropionic acid induced motor impairments and neuroinflammation in rat model of Huntington's disease. *Metab Brain Dis.* **33**, 1459-1470 (2018).

DOI 10.3791/69924

25. Firdaus, F., Zafeer, M. F., Ahmad, M., Afzal, M. Anxiolytic and antiinflammatory role of thymoquinone in arsenicinduced hippocampal toxicity in Wistar rats. *Heliyon.* **4**, e00650. (2018).
26. Abu-Elfotuh, K. et al. The protective effect of thymoquinone or/and thymol against monosodium glutamate-induced attention-deficit/ hyperactivity disorder (ADHD)-like behavior in rats: Modulation of Nrf2/HO-1, TLR4/NF-κB/NLRP3/caspase-1 and Wnt/β-Catenin signaling pathways in rat model. *Biomed. Pharmacother.* **155**, 113799 (2022).
27. Eker, A., Eraslan, G. Single and combined effect of chrysin and N-acetylcysteine against deltamethrin exposure in rats. *Food Chem. Toxicol.* **196**, 115191. (2025).
28. Tekeli, M. Y., Eraslan, G., Bayram, L. Ç., Sarıca, Z. S. Effect of diosmin on lipid peoxidation and organ damage against subacute deltamethrin exposure in rats. *Environ Sci Pollut Res Int.* **28**, 15890-15908. (2021).
29. Armstrong, L. E. et al. Effects of Developmental Deltamethrin Exposure on White Adipose Tissue Gene Expression. *J Biochem Mol Toxicol.* **27** (2), 165-171 (2013).
30. Shi, W., Zhang, D., Wang, L., Sreeharsha, N., Ning, Y. Curcumin synergistically potentiates the protective effect of sitagliptin against chronic deltamethrin nephrotoxicity in rats: Impact on pro-inflammatory cytokines and Nrf2/Ho-1pathway. *J Biochem Mol Toxicol.* **33**, e22386 (2019).
31. Li, M. et al. Protective effects of thymol on deltamethrin-induced toxicity of Channa argus in association with the NF-κB/Nrf2/p53 pathway. *Aquaculture.* **559**, 738429. (2022).
32. Li, H., Shi, N., Wu, S., Huang, X. Effect of deltamethrin on production of free radical and transcription factor Nrf2 in rats' brain tissue. *Chin. J. Ind. Hyg. Occup. Dis.* **27** (10), 593-596 (2009).
33. Li, H. Y., Wu, S. Y., Ma, Q., Shi, N. The pesticide deltamethrin increases free radical production and promotes nuclear translocation of the stress response transcription factor Nrf2 in rat brain. *Toxicol Ind Health.* **27** (7), 579-590 (2011).
34. Aboubakr, M. et al. Antioxidant and Anti-Inflammatory Potential of Thymoquinone and Lycopene Mitigate the Chlorpyrifos-Induced Toxic Neuropathy. *Pharmaceuticals (Basel).* **14**, 940 (2021).
35. Abdel-Daim, M. M. et al. Thymoquinone and diallyl sulfide protect against fipronil-induced oxidative injury in rats. *Environ Sci Pollut Res Int.* **25**, 23909-23916 (2018).
36. Qadri, M. M. et al. Thymoquinone Ameliorates Carfilzomib-Induced Renal Impairment by Modulating Oxidative Stress Markers, Inflammatory/Apoptotic Mediators, and Augmenting Nrf2 in Rats. *Int J Mol Sci.* **24**, 10621 (2023).