

Thymoquinone Ingestions Reversed Inflammation Driven Glia activation and Impaired Cognitive associated Behaviour in Cypermethrin Exposed Rats

Abstract

Background: Pyrethroids pose health risks to humans. Therefore, it is imperative to assess the preventive benefits of thymoquinone against neurotoxicity induced by cypermethrin- in the hippocampal dentate gyrus. **Methods:** Forty male adult Wistar rats with an average weight of 180-200g were randomly allocated to five (5) groups, and each comprising eight rats (n=8 per group). The groups were designated as follows, through oral administrations for 14 days: 0.5ml phosphate- buffered saline (PBS) was given to group one; Group two received 20mg/kg of cypermethrin (CYM); Group three received 10 mg/kg of thymoquinone (THQ); Group four received 20 mg/kg of cypermethrin followed by 10mg/kg of thymoquinone (CYM-10mgTHQ); and Group five received 20 mg/kg and 5mg/kg cypermethrin and thymoquinone respectively (CYM-5 mgTHQ). Behavioral, histological, immunohistochemical, and biochemical analyses were conducted post-treatment. **Results:** Cypermethrin administration caused the rise in pro-inflammatory cytokine TNF- α , Nuclear Factor-kappa B (NF- κ B) and increased expression of astrocytes, microglia, and pro-apoptotic protein Bax. Additionally, cypermethrin reduced levels of anti-inflammatory cytokine IL-10 and acetylcholinesterase (AChE) activity. Cytoarchitectural disruption of dentate gyrus were observed. Cognitive deficits were evident. Thymoquinone treatment attenuated TNF- α and NF- κ B elevation, reduced astrocyte, microglial, and Bax expression, and increased IL-10 and AChE. **Conclusion:** Thymoquinone demonstrated anti-inflammatory and anti-apoptotic effects against cypermethrin-induced neurotoxicity, improving cognitive function in rats.

Keywords: Pyrethroids, Astrocytes, Microglia, Dentate gyrus, NF- κ B.

Introduction

Microglia play a major role in the protection and repair of neurons. However, over-activation of microglia in response to neuronal insult can lead to neuroinflammation, contributing to the progression of neurodegenerative disorders [1, 2, 3]. Prolonged microglial activation results in chronic neuro-inflammation, causing neuronal loss and ultimately neurodegeneration^[4] partly due to the rise in pro-inflammatory cytokines and reactive oxygen species (ROS) ^[5,6].

In recent years, the widespread use of inorganic insecticides, such as pyrethroids, has raised concerns due to their adverse effects on neurological health ^[7,8,9]. Cypermethrin, a type II pyrethroid, is known for its neurotoxic effects, primarily through the prolonged opening of voltage-gated sodium channels (VGSC) ^[10] most insecticides commercially developed act on the sodium channel and the GABA system. Pyrethroids slow the kinetics of both activation and inactivation gates of sodium channels resulting in prolonged openings of individual channels. This causes membrane depolarization, repetitive discharges and synaptic disturbances leading to hyperexcitatory symptoms of poisoning in animals. Only a very small fraction (~1%, and its ability to move across the blood-brain barrier exacerbates its neurotoxicity ^[11]. Cyper-

methrin exposure has been linked with the rise of pro-inflammatory cytokines, TNF- α , IL-1, and apoptotic changes characterized by reduced Bcl2 levels and increased Bax expression ^[12].

Nigella sativa, has a long history of medicinal use and contains thymoquinone (THQ) as its primary active component ^[13,14]. THQ has demonstrated various medicinal properties, including anti-inflammatory and antioxidant effects, making it a promising candidate for neuroprotection ^[15,16]. THQ has been shown to mitigate oxidative and inflammatory damage in brain tissue by down-regulating pro-inflammatory cytokines, inhibiting lipid peroxidation, and preventing apoptosis ^[17]. Moreover, THQ exhibits neuroprotective effects by maintaining mitochondrial membrane potential, preventing dopaminergic neuron degeneration, and reducing excitotoxicity^[18,19].

Given the potential neuroprotective properties of THQ, this study aims to evaluate its effectiveness in mitigating neuroinflammation and associated cognitive deficits induced by cypermethrin exposure.

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Materials and Methods

This study was approved by the University of Ilorin ethical review committee. Approval date 10th June 2021. Approval no UERC/ASN/2021/2137. Identification Code UERC/BMS/182

Experimental Design

This study employed 40 adult male Wistar rats weighing between 180grams and 200 grams. The thymoquinone used in study was acquired from the MedChemExpress (MCE) in the United States of America (Catalog No: HY-d0803), while Ibukun Oluwa Agrochemical Distop based in Ilorin Nigeria sold the 10% cypermethrin (EC) with a reference number ACEC20L068, and accompanied by a NAFDAC Number: A5-0108. The animals were housed in cages within the animal house of the faculty of basic medical sciences, University of Ilorin. These animals were maintained under normal day-night cycles, a standard chow diet was provided, and had access to water freely.

The animals were allocated randomly into five groups, each comprising eight rats ($n=8$ per group). The groups were designated as follows through oral administrations for 14 days: 0.5ml phosphate- buffered saline (PBS) was given to group one; Group two received 20mg/kg of cypermethrin (CYM); Group three received 10 mg/kg of thymoquinone (THQ); Group four received 20 mg/kg of cypermethrin followed by 10mg/kg of thymoquinone (CYM-10mgTHQ); and Group five received 20mg/kg and 5mg/kg cypermethrin and thymoquinone respectively (CYM-5mgTHQ). Following the treatment regimen, behavioral assessments, histological examinations, immunohistochemistry analyses, and biochemical evaluations were conducted. On the 14th of the experiment, the animals were exposed to behavioural studies using Y-maze paradigms and open field test.

Behavioral Assessments

The working memory index (i.e. Assessment of Spatial Memory) of experimental Wistar rats was evaluated using the Y-maze paradigm adapted from^[20], while motor-related behavior was assessed using the open field test maze (OFT)^[21].

Tissue Harvesting

Following the transcatheter perfusion of these animals, the entire brain was removed and subsequently post-fixed overnight in 4% paraformaldehyde. The hippocampal CA regions were then carefully removed and these tissues were placed in a 30% sucrose solution for equilibration. 2 μ m thick sections were obtained from the paraffin-embedded tissue blocks and they were later mounted onto glass slides.

Tissue Staining and Immunostaining Techniques

Hematoxylin and eosin staining techniques was utilized to visualize the entire cellular architectural organization of the hippocampus. Immunohistochemistry was employed to identify astrogliosis, characterized by increased expression of glial fibrillary acidic protein (GFAP). Also, it was used to detect activated microglia using ionized calcium-binding adapter molecule 1 (Iba1). The presence of neuronal cell death was assessed through the detection of Bax expression. The avidin-biotin complex procedure was employed for the immunostaining, with all antibody markers diluted at a ratio of 1:100. The tissue samples were then processed, sectioned to a thickness of two microns

using a rotary microtome, and subjected for 40 minutes to heat treatment at 90°C, to enhance tissue adherence. Quantification of immunopositive cells was performed using the cell counter tool in ImageJ software.

Biochemical Evaluation

The hippocampal tissue was extracted from rats in each experimental group and homogenized in a 0.25M sucrose solution using a mortar and pestle. The obtained homogenate underwent centrifugation at 5000 revolutions per minute for ten minutes. Subsequently, the resulting supernatant was gathered and preserved at -4°C. Following this, an analysis of inflammatory markers including Tumor Necrosis Factor-alpha (TNF- α), Interleukin-10 (IL-10), Nuclear Factor-kappa B (NF- κ B), and Acetylcholinesterase (AChE) was conducted. However, the assays for Tumor Necrosis Factor-alpha, Interleukin-10, and Nuclear Factor-kappa B utilized the sandwich enzyme-linked immunosorbent assay (ELISA) principle, as outlined by Hornbeck^[22]. While A modified Ellman method was employed to determine AChE activity (23). The data obtained were analyzed using GraphPad Prism 8.0, where a four-parameter logistic curve (4PL-curve) was generated and used to extrapolate the values of the samples.

Statistical Analysis

Analysis of the data was conducted with GraphPad Prism version 8.0. To depict the data, the mean and standard error of the mean ($M \pm SEM$) were used. For comparison of mean differences among multiple groups, analysis of variance (ANOVA) was applied. Subsequently, the Tukey post hoc test was employed to ascertain significance at a threshold of $p < 0.05$.

RESULTS

Thymoquinone Improved Cognitive functions following Cypermethrin Toxicity

The CYM group exhibited a significant decrease in percentage alternation (28.40 ± 1.27) compared to the PBS and THQ groups (Fig. A1). Conversely, spontaneous alternation significantly increased in both CYM-LTHQ and CYM-HTHQ groups compared to the CYM group (Fig. 1A). While ambulation in CYM-exposed animals was lower compared to PBS and other experimental groups, this reduction did not reach statistical significance (Fig. 1B). Rearing frequency was higher significantly in the PBS group when compared to CYM. Additionally, there was an increase in rearing frequency in the intervention groups CYM-LTHQ and CYM-HTHQ compared to CYM-exposed animals (Fig. 1C).

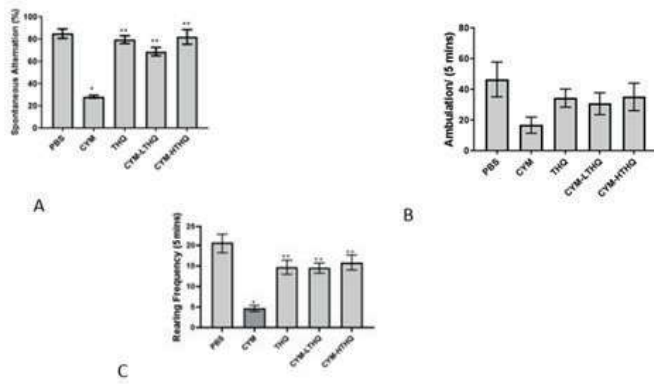


Figure 1: (A): Spontaneous alternation, (B): Ambulation and (C): Rearing frequency.

PBS= Phosphate buffer saline; CYM = Cypermethrin; THQ = thymoquinone; CYM-LTHQ = Cypermethrin before 5 mg of thymoquinone and CYM-HTHQ= Cypermethrin before 10 mg of thymoquinone. Single asterisk (*) indicates significant ($p < 0.05$) compared to PBS, Double asterisk (**) indicates significant ($p < 0.05$) compared to CYM.

Thymoquinone Modulates Inflammatory Responses in Cypermethrin Neurotoxicity

The concentration of TNF- α was higher in the CYM group (12.00 ± 0.86) compared to the other groups: PBS, THQ, CYM-LTHQ, and CYM-HTHQ; however, the difference in these groups were not significant. Subsequently, there was significant reduction in IL-10 levels of CYM group (4.93 ± 0.27) compared to PBS (13.60 ± 2.05), and THQ groups (11.90 ± 1.44). The experimental groups; the CYM-LTHQ and CYM-HTHQ had increased IL-10 levels than in the CYM group, nevertheless, the difference in these groups were not significant. CYM exposure increased Nf-kB concentration significantly to the PBS control and the other experimental groups, except for the CYM-LTHQ group, which was not significant (Table 1).

The AChE activity was significantly lower in CYM group (1886 ± 9.70) as compared to PBS group (2087 ± 32.50), but not significant when compared with the THQ group (2209 ± 81.90). However, there was a significant rise in AChE activities of CYM-LTHQ and CYM-HTHQ groups as compared to the CYM group (Table 1).

Histological and Immunohistochemistry

Observable distortion in the cellular arrangement of the dentate

gyrus and loss of cell shapes were noted in the CYM-treated animals. Additionally, numerous necrotic-like pyknotic cells were seen as well as loss of cells in the granular layer of the CYM-exposed rats (Fig. 2A). THQ post-treatments restored the integrity of the dentate gyrus and reduced the level of observable pyknotic cells. High expression of GFAP was observed in the CYM-treated rats, indicating astrocyte activation in this group of animals (Fig. 2B). Similarly, there was an increase in microglial expression in the dentate gyrus, as evidenced by high Iba-1 expression in the CYM-exposed group of animals (Fig. 2C). Bax-positive cell expression was also high as a result of CYM exposure (Fig. 2D). Post-treatment with THQ was observed to lower the expression of GFAP, Iba-1, and Bax-positive cells (Fig. 2D), thereby reducing the activation of astrocytes, microglia, and apoptotic cell death.

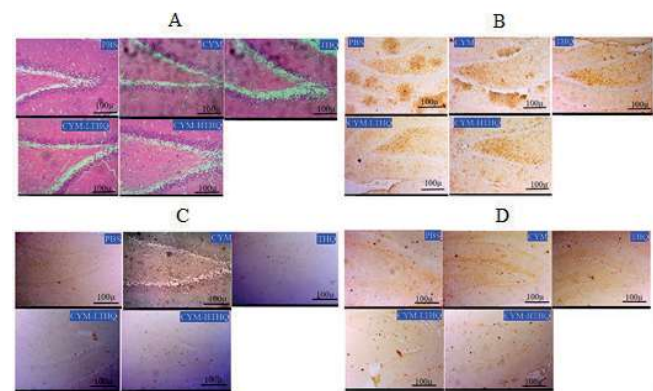


Figure 2: Photomicrograph of dentate gyrus; A- H&E, B- Anti-GFAP, C- Anti-Iba-1, D- Anti-Bax.

PBS- (Phosphate buffered saline); CYM- (Cypermethrin); THQ- (Thymoquinone); CYM-LTHQ- (Cypermethrin before 5 mg/kg of thymoquinone); CYM-HTHQ- (Cypermethrin before 10 mg/kg of thymoquinone). Scale bar 100 μ

DISCUSSION

Inflammation is a common response to disruptions in tissue homeostasis caused by various stimuli such as pathogens, tissue injury, or contaminants, involving the activation of innate and adaptive immunity [24,25]. Cognitive impairment is a prevalent manifestation of neurodegenerative diseases like Alzheimer's and Parkinson's diseases, highlighting the urgent need for therapeutic drugs to counteract the neurodegenerative processes associated with cognitive deficits. Mitochondrial dysfunction,

Table1: Effects of Thymoquinone on Cypermethrin-induced inflammatory responses and Acetylcholinesterase activity in adult male Wistar rats

Groups N=6	TNF- α (pg/mL)	IL-10 (pg/mL)	NF-kB (ng/mL)	AChEs (unit/L)
PBS	6.82 \pm 1.40	13.60 \pm 2.05	1.40 \pm 0.12	2087 \pm 32.50
CYM	12.00 \pm 0.86	4.93 \pm 0.27*	2.20 \pm 0.11*	1889 \pm 9.70*
THQ	9.64 \pm 2.89	11.90 \pm 1.44**	1.04 \pm 0.17**	2209 \pm 81.90
CYM-LTHQ	8.31 \pm 1.00	7.68 \pm 1.56	1.90 \pm 0.20	2393 \pm 89.40
CYM-HTHQ	7.33 \pm 0.89	7.16 \pm 1.56	1.51 \pm 0.16**	2626 \pm 43.30**

CYM-(Cypermethrin); THQ-(Thymoquinone); CYM-LTHQ-(Cypermethrin before 5mg/kgbw of thymoquinone); CYM-HTHQ-(Cypermethrin before 10mg/kgbw of thymoquinone).a,b,c showing significant difference from PBS, CYM and THQ respectively at $p < 0.05$.

neuroinflammation, oxidative stress, autophagic-lysosomal cascade alterations, and excitotoxicity, are all known to play critical roles in the process of neurodegenerative diseases. Acetylcholine (ACh) is a crucial neurotransmitter that regulates learning and memory; its inhibition can lead to learning and memory deficits as well as impairments in motor functions [21,26,27].

In this study, exposure to cypermethrin markedly reduced AChE activity, a typical effect of insecticides, and likely attributable to the potent neurotoxic effects of cypermethrin. Pyrethroids like organophosphates and other pesticides act as AChE-specific inhibitors due to their binding affinity for the enzyme's ester site, leading to its inactivation [28,29]. However, although AChE inhibitors are crucial in managing memory deficits, CYM-induced inhibition of AChE did not enhance learning and memory in the experimental rats, likely due to the induction of inflammation and neuronal damage by CYM, established causes of cognitive impairment. AChE inhibition caused by CYM and other insecticides is a long-term effect. An increase in AChE activity would likely reduce the concentration of acetylcholine neurotransmitter, which is detrimental to learning and memory [27]. However, it might lead to reduced motor and anxiety-related behaviors such as muscular paralysis, convulsions, bronchial constriction, and death by asphyxiation [27]. Induction of inflammation and loss of dentate gyrus neuronal integrity are important factors contributing to the spatial memory loss observed in this study. Thymoquinone intervention modulated neuroinflammation, which caused the reduction in ROS generation and improved dentate gyrus cytoarchitecture, thereby enhancing memory and motor functions. These findings align with those of Kassab & El-Hennamy, [30], who reported increases in AChE and ATPase activities in various brain regions of rats following exposure to arsenic.

Anti-inflammatory therapy has proven beneficial against many diseases of microglial activation, ROS generation and inflammation origins [31,32]. In this study, thymoquinone modulated inflammatory-mediated cognitive deficits by deactivating NF- κ B. NF- κ B is a regulatory protein whose nuclear translocation, following phosphorylation of its inhibitory agent I κ B, induces the proliferation of pro-inflammatory cytokines; IL-1, TNF- α , COX-2, iNOS, and vascular adhesion molecules. Prolonged TNF- α release due to persistent NF- κ B activation leads to compensatory increases in cellular ROS generation. Excessive ROS generation leads to decreased expression and activity of antioxidant enzymes. Exposure of experimental rats to cypermethrin caused increased levels of the NF- κ B transcription factor due to phosphorylation of the I κ B inhibitory factor, eventually leading to the stimulation of transcription and release of tumor necrosis factor- α , as observed in this study. However, the anti-inflammatory cytokine level was low due to cypermethrin exposure, explaining its potential as a neurotoxicant that induces toxicity, including inflammation. Previous research by Singh *et al.*, [33] and Tiwari *et al.*, [34] found that cypermethrin exposure caused the increase of prominent pro-inflammatory protein IL-1 in the striatum of adult rats, as well as increased levels of TNF- α in the substantia nigra and striatum, respectively. The findings revealed that administration of thymoquinone at two separate doses of 5 mg/kg and 10 mg/kg respectively; body weights reduce the concentration of transcription factor NF- κ B, indicating an inhibitory action of thymoquinone on NF- κ B. This suggests that thymoquinone exerted its anti-inflammatory role on cypermethrin-induced neurotoxicity by deactivating NF- κ B, leading

to a decrease in TNF- α levels and subsequent increase in IL-10 levels.

The anti-inflammatory effects of thymoquinone observed can also be attributed to its ability to inhibit the phosphorylation of I κ B, as phosphorylation of I κ B enables the activation of NF- κ B, allowing its translocation into the nucleus where it initiates the downstream transcription of inflammatory proteins [35,36]. Also, Wang *et al.*, [37] reported the anti-inflammatory role of thymoquinone, where they found that thymoquinone inhibited IL-1 β , TNF- α , NO, and PGE2 production, as well as suppressing NF- κ B.

Environmental toxins and heavy metals have been shown to cause mild to severe damage in neuronal cyto-architectures, such as pyknotic nuclei and neuronal cell shape deformation. In this study, cypermethrin caused disorganization of the granular layer in the dentate gyrus, with pyknotic cells, vacuolated cells, and a loss of granule cell spherical shape. The evident pyknosis, resulting from DNA fragmentation and chromatin condensation causing nuclear disintegration and vacuolization, indicates that dentate gyrus and hippocampal neurons undergo degeneration from CYM neurotoxicity. This loss of morphological integrity in the granule cells due to cypermethrin exposure may be attributed to its lipophilic nature, enabling CYM, like other pyrethroids, to cross the blood-brain barrier, where it alters sodium channels in the nerve membrane Sallam *et al.*, [38]. Because the dentate gyrus is crucial in hippocampal formation, a disruption in its morphological integrity undoubtedly contributes to the development and progression of memory loss, changes in perception, and training, and a reduction in recollection capacity [39]. This strengthens the observed loss of cognitive function due to cypermethrin despite a reduce AChE activity. Latuszynska *et al.*, [40] and Cao *et al.*, [41], reported focal pyknosis in the cortex cerebri and concentrations of neurocytes in the cytoplasm of the stratum granulosum, hypothalamus, and cerebral cortex following exposure of rats to a mixture of CYM and chlorpyrifos, were in line with this study. Additionally, Ahmad *et al.*, [42] reported graded degeneration of pyramidal neurons in the cerebral cortex and Purkinje cell degeneration in the cerebellar cortex of rabbits due to CYM administration.

Thymoquinone preserved the histoarchitecture and function of the dentate gyrus against the neurodegenerative effects of CYM. It also reduced the extent of vacuolated cells and chromatin condensation, thereby contributing to improved neuronal connections of the hippocampal formation. Since lesions in the dentate granule cells result in memory loss, restoring the lesions is crucial for restoring memory impairment. Nigella sativa oil has been reported to improve the histoarchitecture of the brain by reducing neuronal cell degeneration in various brain regions following dichlorvos exposure [26]. In the study by Imam *et al.*, (43), Nigella sativa oil, the parent compound of thymoquinone, preserved cerebellar Purkinje cells after aluminum chloride administration. The findings of this study are supported by those of Adana *et al.*, [44], who reported that thymoquinone preserved hepatic cytoarchitecture in rats following cyclophosphamide administration.

Astrocyte and microglial activation following CYM exposure were observed in the dentate gyrus. Astrocyte activation induces microglial cells to start secreting various immune response factors, such as pro-inflammatory cytokines, chemokines, cy-

totoxic factors, and mitochondrial fragmentation, causing a corresponding increase in astrocyte activation^[45,46,47]. The increase in the NF- κ B and TNF- α levels seen in this study may be due to gliosis caused by CYM administration, as microglial activation increases the production of inflammatory cytokines, the stimulation astrocytes indicates increased ROS generation. Thymoquinone modulated the regular functions of astrocytes and microglia by reducing their hyperactivity, decreasing NF- κ B levels, and corresponding TNF- α levels, likely attributable to THQ's potent anti-inflammatory and antioxidant effects. Since increased microglial activation leads to increased inflammatory cytokines and ROS generation^[48], a reduction in microglial activation due to THQ, can also be linked to a decrease in the TNF- α levels, and the increase in IL-10 levels.

The effects of cypermethrin induced an increase in the proliferation of the pro-apoptotic protein Bax, as well as a marked increase in Bax immunopositive cells. Cypermethrin induces apoptosis in the rat brain through the generation of ROS and cytotoxins, mitochondrial damage, the cytochrome c release, and caspases 3 and 9 activation; these are important in extrinsic and intrinsic process of apoptosis. Pandey *et al.*,^[49] showed that cypermethrin administration caused increased expression of P53 and decreased Bcl-2 levels by inducing miR-200 and apoptosis in neuronal cells. Raszewski *et al.*,^[50] reported that cypermethrin and chlorpyrifos can induce apoptosis in human's neuroblastoma cell line SH-SY5Y. Stressing that, they induce apoptosis by increasing the activation.caspase-3. Singh *et al.*,^[33] and Agrawal *et al.*,^[51] reported that cypermethrin caused mitochondrial damage and can lead to increased levels of the following enzymes which include; cytochrome c, activation of caspase-3, as well as the increased expression of Bax and COX-2, and P53 protein.

The increased Bax expression levels following exposure to cypermethrin in this study could be due to damage to mitochondria and a subsequent increase in free radical generation, as cypermethrin has been shown to disrupt mitochondrial integrity and increase ROS generation. Similar to the observation of this study, Pandey *et al.*,^[48] demonstrated that cypermethrin treatment raised P53 expression and lowered Bcl-2 levels in neural cells by inducing miR-200 and death. Raszewski *et al.*,^[49] discovered that cypermethrin and chlorpyrifos increase caspase-3 activation, triggering apoptosis. Singh *et al.*,^[33] found that cypermethrin caused mitochondrial damage, resulting in higher levels of cytochrome c, activating caspase-3, elevating COX-2 protein. Thymoquinone reduced Bax immunopositive cell expression, inhibiting its apoptotic action, suppressing its oligomerization, and inhibiting mitochondrial release of apoptogenic chemicals. Also, the antiapoptotic activity of thymoquinone may directly block cytochrome c production and hence inhibit the adaptor molecule APAF-1 and activation of caspase-9. Thymoquinone also acts as an anti-proliferative agent and regulates apoptosis in cancer progression by decreasing Bcl-2 expression, increasing Bax/BAD levels, and inducing tumor and cancer cell apoptosis and autophagy. Thymoquinone suppresses the growth of malignancies in various organs, such as the prostate and breast. An earlier study found that thymoquinone ingestion decreased the expression of both Bcl-2 and p53 genes, however, there was an observable increment in the expression of the Bax/BAD gene in MCF-7 cells; but in non-cancer HEK293 cells, there was an increase expression of Bcl-2 and p53 genes and decrease in the proliferation of Bax/BAD genes. Thymoquinone has been prov-

en to protect the cortex of rats against acrylamide-induced neurotoxicity via MAP kinase signaling pathways.

CONCLUSION

In conclusion, this study underscores the detrimental actions of cypermethrin exposure on cognitive function and neuronal integrity in experimental rats. Cypermethrin induced significant alterations in AChE activity, inflammatory cytokine levels, dentate gyrus morphology, and apoptotic protein expression, which have all contributed to the cognitive deficits and neuronal damage.

However, thymoquinone (THQ) demonstrated promising neuroprotective effects against cypermethrin-induced toxicity. THQ treatment mitigated the reduction in AChE activity, attenuated inflammatory responses by modulating NF- κ B signaling and cytokine levels, preserved dentate gyrus histoarchitecture and inhibited apoptotic pathways. These findings suggest that THQ holds therapeutic potential in mitigating the neurodegenerative effects associated with pesticide exposure.

Patient informed consent: There is no need for patient informed consent

Ethical committee approval: This study was approved by the University of Ilorin ethical review committee. Approval date 10th June 2021. Approval no UERC/ASN/2021/2137. Identification Code UERC/BMS/182

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Author contribution subject and rate

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Fatimo Ajoke Sulamon: 10%: study analysis and interpretation

Aminu Imam: 15%: Study analysis and interpretation

Ruqayyah Yetunde Ibiyeye: 10%: Data collection

Misturah Yetunde Adana: 10% critical review of the manuscript

Omoola Olasheu Oluwatosin: 10%: Referencing

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