Quest Journals
Journal of Medical and Dental Science Research
Volume 12~ Issue 6 (June 2025) pp: 06-18
ISSN(Online): 2394-076X ISSN (Print):2394-0751
www.questjournals.org

Research Paper

Therapeutic potential of *Zingiber officinale* methanol extract in $CuSO_4$-induced behavioral deficit and hippocampal redo-inflammation associated with Nrf2 downregulation in mice

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Abstract: *Disturbances in neuroimmune system have significant implication in eliciting cognitive and neurobehavioral deficit, and biochemical changes associated with $CuSO_4$-induced neurotoxicity. Plant-based food rich in phytochemical property is suggested to inhibit the mechanism implicated in $CuSO_4$-induced neurotoxicity. Thus, this study investigated the mechanisms through which Zingiber officinale Roscoe methanol extract (MEZO) exerts its therapeutic effects after $CuSO_4$ exposure in mice. Male mice were randomly divided into five experimental groups (n = 8) including a control group administered distilled water (10 mL/kg, p.o), $CuSO_4$-exposed group (20 mg/kg, i.p), and MEZO (50 and 100 mL/kg, p.o) and Vit. C (100 mL/kg, p.o) treated groups. MEZO and Vit. C treatment was done after 1 hr of daily exposure to $CuSO_4$ for 28 days. Cognitive functions and phenotypes of neurobehavioral impairment, markers of oxidative stress, inflammatory mediators, neurochemical transmission and hippocampal CA3 subfield were evaluated. The result showed that following $CuSO_4$ assault, MEZO treatment prevent the reduction in locomotor activity and improved the spatial memory deficit and anxiety- and depressive-like behavior in mice. MEZO treatment modulated the hippocampal neuroimmune system by inhibiting oxidants, pro-inflammatory mediators and inflammatory enzyme activities, and improving the release of endogenous antioxidant enzymes associated with Nrf2 upregulation. Further, MEZO enhances cholinergic transmission by suppressing the AChE activity as well as abated the loss of hippocampal CA3 subfield. The endpoint of the study suggests that MEZO exact a neuroprotective effect by targeting the redo-inflammatory pathway associated with upregulation of Nrf2 protein.*

Keywords: *Copper II sulfate; Oxidative stress; Inflammation; Zingiber officinale; Neurobehavior*

***R**eceived 01 June., 2025; **R**evised 06 June., 2025; **A**ccepted 08 June., 2025 © The author(s) 2025.*
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I.INTRODUCTION

The involvement of hippocampus in episodic memory formation and recall of personal and semantic experiences is well-documented[1-2]. Inflammation, altered energy metabolism, synaptic and neuronal loss, aberrant protein aggregation and proteostasis in the hippocampal are identified as the hallmarks of neurodegenerative diseases (NDDs) associated with behavioral impairment which include Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and many others[3-6]. Of note, the propensity of hippocampal injury evolves into depression, cognitive and emotional dysregulation[7-9]. Hippocampal damage has been linked to the activation of important pathways that mediate neuroinflammation indicated by the level

of inflammatory factors like IL-6, TNF-α, NF-κB and COX2[10]. Moreover, exposure to environment insults such as metals and other pathogens affect the central nervous system, precisely the brain and its associated structures.

Copper (Cu) is a well-known micronutrient that is involved in several physiological processes including wound repair, connecting tissue formation, antioxidant defense, mitochondrial respiration, and the modulation of a variety of enzyme activities that catalyzes biological compound synthesis [11-12]. In the brain, Cu is maintained at a relatively low concentration, and it is believed to play a key role in regulating neurotransmitter synthesis, myelination of neurons and synaptic transmission[11]. Meanwhile, exposure to excess Cu may occur due to inborn defects of Cu metabolism, environmental contamination or occupational hazards[13]. Abnormal brain copper level (Cu) affects neuronal functions contributing to neurodegenerative disease pathology [13,14]. It is characterized by synapse neural network loss, neurofibrillary tangles and neuritic plaques[15-16, 10]. Cu overload has been reported to compromise learning and memory and provoke hippocampal oxidative stress in rats[14]. This metal exposure, above its acceptable limit, has been linked to alter serotonergic, dopaminergic, glutaminergic, glutamatergic, GABAergic and cholinergic transmission[17-20].

Tropical plants have indicated very strong anti-oxidant and neuroprotective, and neurorestortaive properties [15, 16]. Among the numerously consumed and neutraceutically used plants to improve or enhance the body's defense system is the *Zingiber officinale* Roscoe (Zingiberaceae) found in ginger rhizome. It is a widely used folk medicine in common illnesses, including emesis, headaches, colds, and nausea[21-22]. Phytochemical screening has reported the presence of bioactive substances, including phenolic constituents (paradols, shogaols, gingerols) and terpene chemicals in *Zingiber officinale* Roscoe extract [23,21,24]. Recent investigations on the plant have demonstrated that it possesses antioxidants, anti-inflammatory, antibacterial, antimicrobial, neuroprotective, cardioprotective, respiratory protective , anti-diabetic, anti-obesity, antiemetic, and anti-apoptotic properties[25,22]. *Zingiber officinale* antioxidant qualities have been linked to its ability to enhance the endogenous antioxidant enzyme signaling pathway activation[26]. 6-Gingerol, a major gingerol in ginger rhizomes has been shown to ameliorate Aβ-amyloid-induced neurotoxicity in pheochromocytoma (PC12) cultured cells by suppressing reactive oxygen species and inflammatory responses, overwhelming the activation of GSK-3β and improving the activation of Akt[27]. *Zingiber officinale* has been reported to cause suppression of pro-inflammatory cytokines and othe inflammatory mediators such as TNF-α, IL-1β, IL-6, COX-2, and NO in LPS-induced RAW 264.7 cells[28].

However, the role of *Zingiber officinale* against copper-induced hippocampal degeneration and the behavioral deficit is not yet fully understood. The mechanism of *Zingiber officinale* involving the modulation of cholinergic signaling and Nrf2 levels following neuronal insult with copper toxicity remains uncovered. This study seeks to address the question of whether *Zingiber officinale* methanol extract (MEZO) has a neuroprotective effect against CuSO4-induced behavioral deficit and hippocampal redo-inflammation associated with downregulation of Nrf2 proteins in mice.

II. Materials and Methods

Reagents and chemicals

Reagents and compounds used for the study were obtained from Sigma-Aldrich (St. Louis, USA) and Thermo Fisher Scientific, UK, except otherwise specified.

Animal procurement

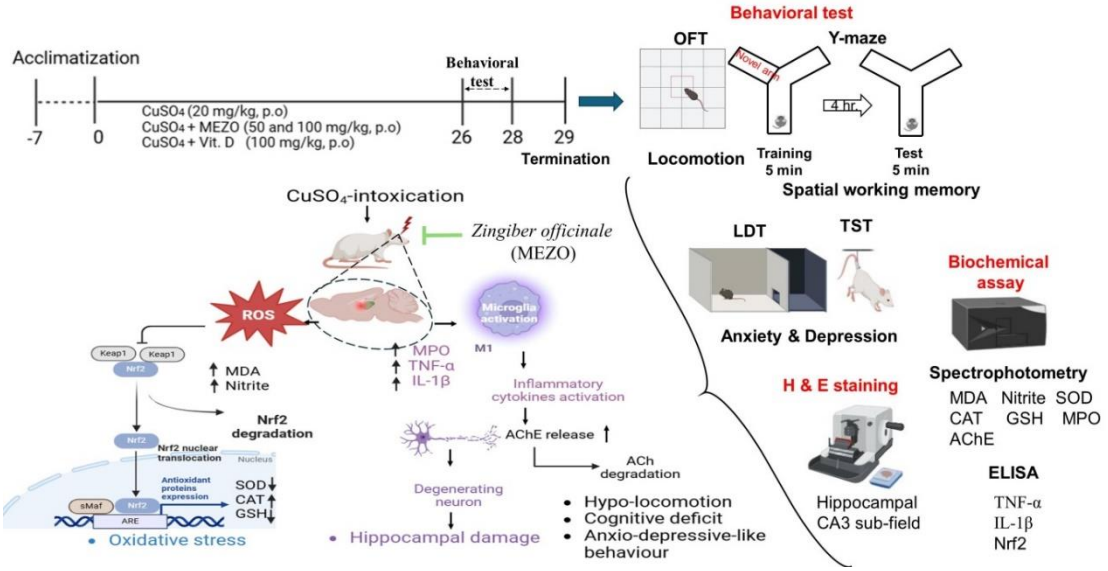
Male Swiss mice weighing 25–27 g (10-12 weeks, N = 40) was obtained from Bayelsa Medical University, Yenagoa, Bayelsa State. The experimental animals were acclimatized in the University Animal Housing Facility under standard laboratory conditions, as specified in NIH publications volume 25 No.28, revised in 1996 and issued ethical approval with the number: BAREC/App/00/012/04 by Bayelsa Medical University animal care and use committee before commencing the study.

Plant collection, identification and preparation

Fresh rhizomes of *Zingiber officinale* were purchased from Eleme, Oil-mill market, Rumukwurushi, Port Harcourt, Rivers State, Nigeria. The rhizomes were authenticated at the Forestry Research Institute of Nigeria, Ibadan (FRIN)-FHI 110038, processed into powder form and macerated (300 g, powder) in 1041 ml of hydro-methanol solvent, 99.8 % methanol (ThermoFisher Scientific, UK; Lot No: 1914578) plus distilled water (70:30) for 72 h at room temperature. Using Whatman® qualitative filter paper, Grade No: 1 (Cat No: 1001125) a filtrate was obtained by thrice repeated filtration to achieve a complete extraction process. The resulting mixture was further concentrated using a rotary vacuum evaporator at 40°C under reduced pressure (700 mmHg) to remove solvent from the fraction and obtain a Zingiber officinale methanol extract (MEZO). The final yield of the soaked extract was 22.37 g and the acute lethal toxicity (LD50) of the crude extract was done by the Lorke method[29] as previously reported in our earlier studies[30, 26].

Experimental design

The selected dose for MEZO and Copper (II) sulfate were in line with our previous reports and preliminary studies[30, 26]. The mice were randomly assigned into 5 groups (n = 8) and treated daily for 28 days as follows: Group 1 (normal control): received distilled water treatment (10 mL/kg), Group 2 (negative control): received $CUSO_4$ treatment (20 mg/kg), Group 3 were pre-treated with CUSO4 (20 mg/kg) and received 50 mg/kg of MEZO, Group 4 were pre-treated with CUSO4 (20 mg/kg) and received 100 mg/kg of MEZO and Group 5 were pre-treated with CUSO4 (20 mg/kg) and received 100 mg/kg of Vit C. The co-treated mouse cohort was pretreated with $CUSO_4$ dissolved in distilled water for 1 hr before receiving MEZO (50 and 100 mg/kg) or vitamin C (100 mg/kg) dissolved in distilled water. $CUSO_4$, MEZO and Vit. C were freshly prepared daily and administered by oral gavage (*per os*) (Schematic representation).



Behavioral test

All the experimental mice were subjected to behavioral testing to determine the locomotive performance, spatial memory function and anxio-depressive-like behavior after $CUSO_4$ lesioning on days 26, 27 and 28 using open field apparatus (OFT), Y-maze, light and dark transition test (LDT) and tail suspension test (TST). The behavioral tests were monitored by two trained individuals who were not aware of the treatment conditions or the research design of the study.

Assessment of locomotion

All the mice were acclimated in the behavioral test room for 1 h, after treatment before the test day 26 to avoid any outcome of environmental stress. Using the open field test (OFT) the effect of MEZO was investigated on locomotor behavior in the $CUSO_4$ lesioned mice as each mouse was individually placed in the center of the OF space (35 × 30 × 23 cm) divided into 3 concentric zones. The number of lines crossed with the entire four limbs was recorded for 5 min as an indicator of spontaneous ambulation[31]. The OF chamber was cleaned with 70% ethanol to prevent olfactory cues from previously tested mouse.

Assessment of spatial memory function

To assess the short-term spatial memory function of the $CUSO_4$ lesioned mice after MEZO administration, the Y-maze test was adopted on day 27. Each mouse was placed in the Y-maze apparatus of three arms (15 x 5 x 10 cm) labeled ABC at 120°. In brief, each mouse was trained with one arm (C quadrant) closed off on the Y-maze apparatus restricting the mouse to arms A and B for familiarization for 5 min[32]. 4 hours after each experimental mouse training phase, the mice were allowed free access to the third arms and tested for 5 min. The total time taken for each mouse in the center, A, B and C quadrant, was recorded for 5 min. The Y-maze apparatus was cleaned with 70% ethanol to prevent olfactory cues from previously tested mice.

Assessment of Anxio-depressive-like behavior

The anxiogenic and depressive-like behavior was determined by using the light and dark transition test (LDT) and the tail suspension test (TST) with little modification on days 28. The LDT was conducted using a two-sectioned wooden light-dark apparatus (21 × 42 × 25 cm), having a limited opening of 3 cm x 5 cm[31]. Each rat was placed in the light chamber and allowed to explore the light and dark transition box freely for five

minutes. The time spent in the light and dark zone of the LDT apparatus was used as the criterion for evaluating the anxiolytic treatment effect of MEZO following $CUSO_4$ lesioning in the study. Following each mouse test, the olfactory cues were eliminated using 70% ethanol.

Four hours after the light and dark transition test, the tail suspension test was used to measure the immobility time of each animal. The test assesses the depressive-like behavior in each mouse treated with MEZO or Vit C after $CUSO_4$ lesioning. Briefly, the mice were suspended individually 50 cm above the ground level and approximately 1cm from the tip of the tail for 6 min with their tail held with masking tape. The mice were acclimatized on the TST apparatus for the first 2 min, while the duration of immobility (freezing) was recorded in the last 4 min. We considered each mouse immobile when it remained passive and completely motionless[11].

Biochemical and histological analysis

The mice were anesthetized with ketamine hydrochloride and then euthanasia by cervical dislocation. For the biochemical assays, five mice were selected. The brains was isolated and then dissected to obtain the hippocampus. The dissected hippocampi were instantly homogenized in cold phosphate buffer (PBS) (10% w/v, 0.1 M, pH 7.4) and centrifuge for 10 minutes at 10,000 rpm and 4 ºC, the supernatant was kept at -20 ºC until analysis. Furthermore, transcardial perfusion with PBS and 10% neutral buffered formalin was done on three mice before the brains were removed for histological processing.

Estimation of oxidative stress markers

According to the guidelines, the hippocampal oxidative stress marker was evaluated. First, the using Gornall et al. method hippocampal protein level was computed. Lipid peroxidation level was determined by evaluating the malondialdehyde content (MDA)[33-34] and nitrite level by using Greiss reagent[35]. Antioxidant enzyme activity such as catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH) level were assessed as earlier develope[36-38], respectively. The entire hippocampal oxidative stress markers were estimated by spectrophotometric method.

Estimation of acetylcholinesterase levels

According to[39], acetylcholinesterase activity was determined by spectrophotometric method. Briefly, an aliquot of 0.4 mL of the hippocampal supernatant was used and mixed with phosphate buffer (2.6 mL; 0.1 mol/L; pH 7.4) and DTNB (0.1 mL). 0.1 mL of acetylthiocholine iodide solution was added to the reaction mixture and the absorbance was read using a UV/Vis spectrophotometer (INESA) at 412 nm for three minutes at one-minute intervals. AChE activity levels were computed and represented as mmol/min/mg protein after the absorbance change per minute was determined.

Estimation of myeloperoxidase levels

According to the Bradley et al. method, the hippocampal myeloperoxidase level was measured [40]. In extraction buffer (0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer at pH 6.0), the hippocampal homogenates obtained were suspended and frozen at 20 °C and freeze-thawed three times before centrifuging the suspension for roughly 15 minutes at 15,000 rpm and 4 °C. To assess the MPO activity, 0.2 mL of the supernatant was added to a mixed solution containing 0.15 mM H_2O_2 (2.8 mL) and 0.167 mg/mL O-dianisidine in 50 mM potassium phosphate buffer. The UV/Vis spectrophotometer (INESA) was used to track the change in absorbance set at 450 nm over 3 minutes. One MPO unit was defined as the change in absorbance at 0.001/min with the specific activity considered as the unit of MPO/mg of protein.

Estimation of pro-inflammatory cytokines and nuclear factor erythroid 2-related factor 2

TNF-α, IL-1β (BioLegend, USA) & Nrf2 (Elabscience, USA) in the hippocampus were done at room temperature according to the ELISA kit manufacturer's procedure with the protein content obtained from the kits' standard curve. Readings were done with a 96-well ELISA microplate reader (Sunnyvale, CA) and expressed as pg/mg protein.

Histological examination

The brain tissues were excised after trans-cardiac perfusion with normal saline (0.9 %) followed by a preservation process in 10% neutral buffered formalin (NBF) at pH 7.2. Later, the NBF preserved brain tissues were processed and sliced transversely to obtain 5 µm sections with the microtome. The sections were then stained in haematoxylin and eosin (H&E) solution to detect cellular condensation, vacuolation and necrosis[41]

Statistical Analysis and Image Processing

Graph Pad Prism 8.4.3 (GraphPad Software Inc., San Diego, CA, USA) statistical software and Image-J software (NIH, USA) were used for data analysis, image processing, and neuronal quantification respectively. Comparison of experimental groups with analysis of variance (ANOVA) and Tukey post hoc test was used and presented as means ± S.E. with the $p < 0.05$ considered significant. The captured images were processed with cell count plugin on the Image J software by two trained scientist blinded to the study.

III. Results

Effect of *Zingiber officinale* methanol extract on behavioral outcomes in $CuSO_4$-induced behavioral deficit in mice

The effect of *Zingiber officinale* on behavioral characteristics in $CuSO_4$-induced hippocampal behavioral deficit in mice is depicted in fig. 1 A-D. $CuSO_4$ exposed mice demonstrated hypo-locomotion typified by a significant decrease in the number of line crossing [$F(4, 25) = 33.53$, $p < 0.0001$, fig. 1A] in OFT; impaired spatial short-term memory function indicated time spent in familiar quadrants (A and B) [$F(4, 20) = 0.7678$, $p = 0.5587$, fig. 1B], significant increase in time spent in the centre of the quadrant [$F(4, 20) = 60.43$, $p < 0.0001$, fig. 1C] and a decrease in time spent in novel quadrant (C) [$F(4, 20) = 12.96$, $0 < 0.0001$, fig. 1D] in Y-maze test; an increase anxiety-like behavior shown by a decrease in time spent in the light zone and increased in time spent in the dark zone (Treatment: [F4, 50] = 0.000; $p < 0.9999$, Interaction: [F1, 50] = 74.39; $p < 0.0001$ and Interaction × treatment: [F4, 50] = 50.58; $p < 0.0001$, fig. 1E) in light and dark transition test; and an increase in depressive-like behavior indicated by elevated immobility time [$F(4, 25) = 16.87$, $p < 0.0001$, fig. 1F] in TST when compared to control. On the other hand, MEZO treatment reversed hypolocomotion, impaired spatial short-term memory function, and anxio-depressive-like behavior caused by CuSO4.

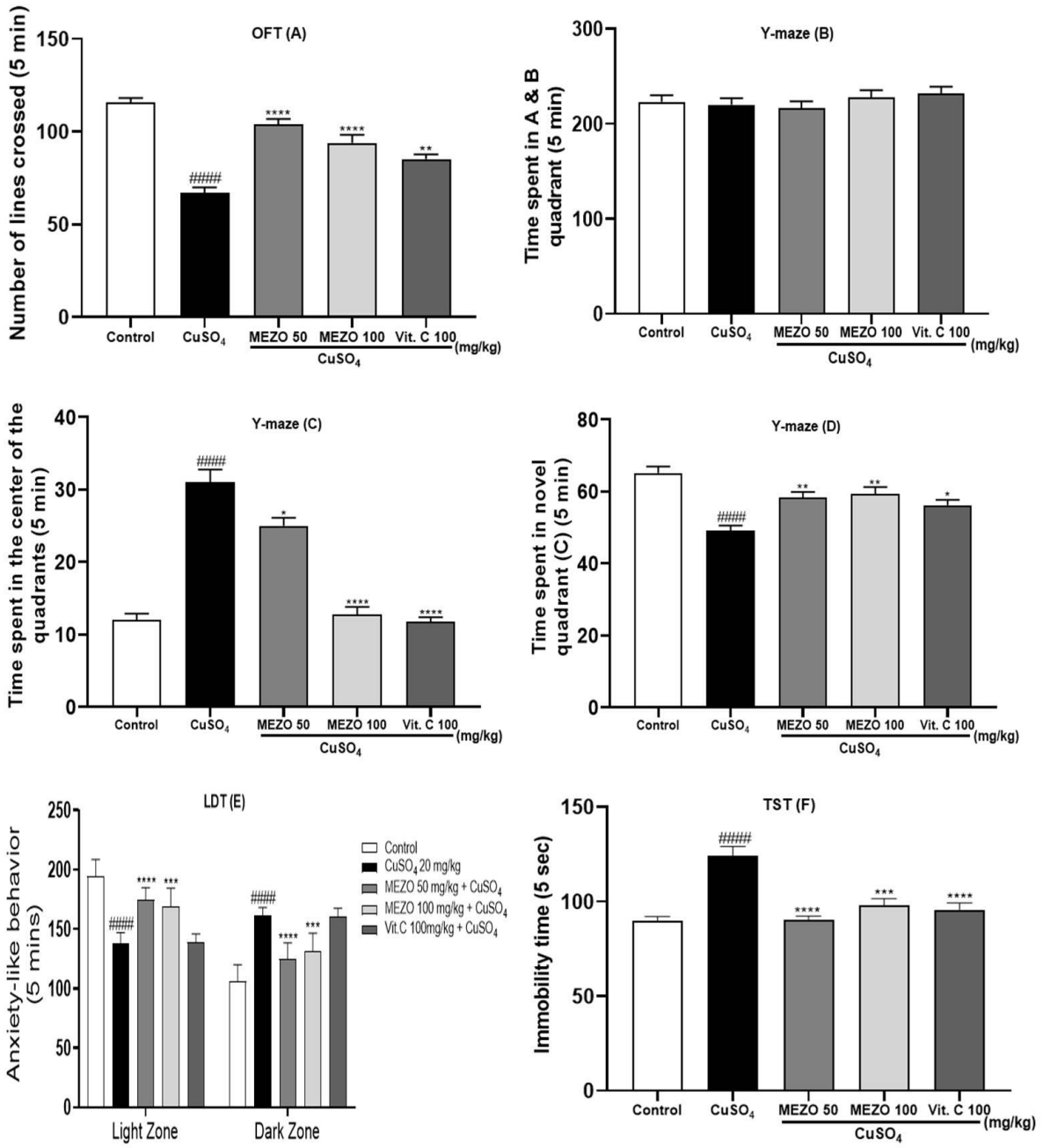


Figure 1 A-E: The Effect of *Zingiber officinale* methanol extract on behavioral outcomes in $CuSO_4$-induced behavioral deficit in mice. (A) OFT, (B-D) Y-maze test (E) LDT (F) TST. Data are presented as mean±SEM (n=8), *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001 vs $CUSO_4$, and $^{\#\#\#\#}$p< 0.0001 vs control. OFT: Open field test, LDT: Light and dark transition test, TST: Tail suspension test.

Effect of *Zingiber officinale* methanol extract on lipid peroxidation and nitrergic activity in $CuSO_4$-induced hippocampal neurotoxicity in mice

The effect of *Zingiber officinale* on oxidative stress in CuSO4-induced hippocampal neurotoxicity in mice is represented in fig. 2 A-B. CuSO4 exposure elevated lipid peroxidation and nitrergic activities indicated by a significant increase in hippocampal MDA [F (4, 10) = 8.010, p = 0.0037, fig. 2A] and nitrite level [F (4, 10) = 9.005, p = 0.0024 fig. 2B] when compared to control. However, MEZO treatment did not reduce the increase in MDA level, but a significantly decreased hippocampal nitrite level with MEZO 50 mg/kg dose and Vit. C 100 mg/kg relative to the untreated CuSO4 exposed mice was observed.

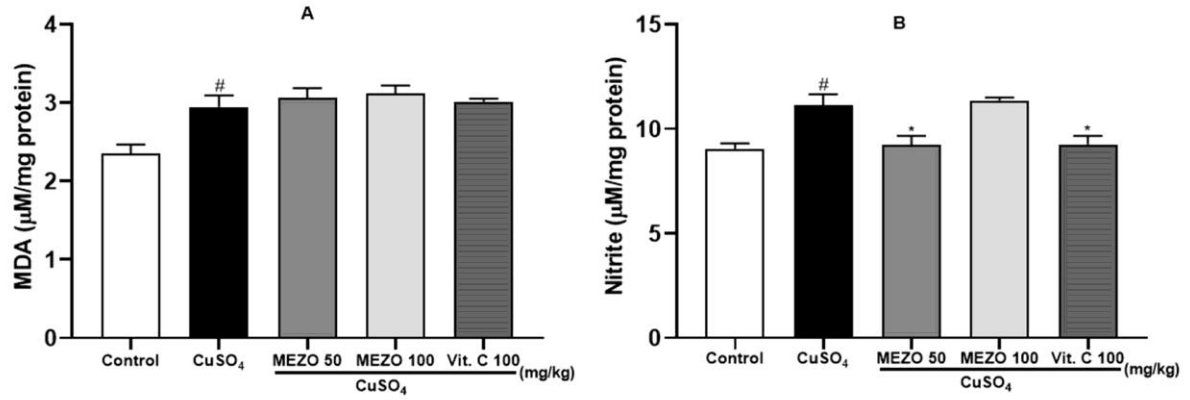


Figure 2A and 2B: Effect of *Zingiber officinale* methanol extract lipid peroxidation and nitrergic activity in $CuSO_4$-induced hippocampal neurotoxicity in mice (A) MDA (B) Nitrite. Data are presented as mean±SEM (n=5), *p< 0.05, vs $CUSO_4$, and #p< 0.05 vs control. MDA: Malondialdehyde.

Effect of *Zingiber officinale* methanol extract on endogenous antioxidant enzymes in $CuSO_4$-induced hippocampal neurotoxicity in mice

As indicated in fig. 3 A-C, CuSO4 exposure reduced hippocampal GSH [F (4, 10) = 14.62, p = 0.0003, fig. 3A] and SOD concentrations [F (4, 10) = 158.6, p < 0.0001, fig. 3C] significantly, while CAT concentration [F (4, 10) = 8.582, p = 0.0028, fig. 3B] significantly increased when compared to control. However, treatment with MEZO improved the antioxidant defense system by increasing the hippocampal GSH (50 mg/kg) and SOD (50 and 100 mg/kg) concentration relative to untreated CuSO4 exposed mice. In addition, the hippocampal CAT concentration (100 mg/kg) decreased relative to untreated CuSO4 exposed mice. Vit. C 100 mg/kg treatment reduced CAT and increased SOD concentration compared to untreated CuSO4 exposed mice.

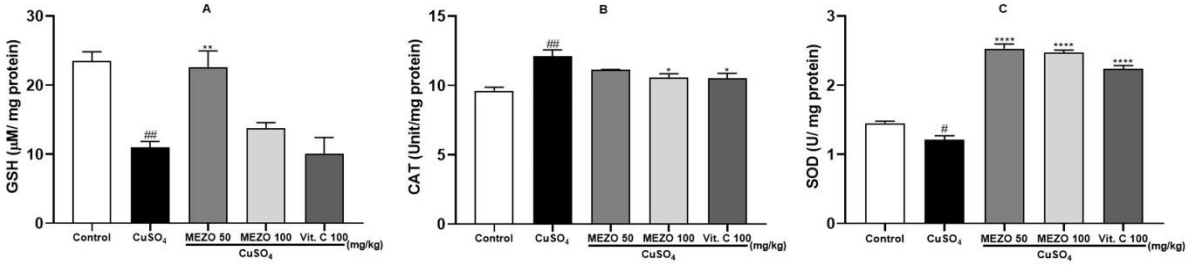


Figure 3 A-C: Effect of *Zingiber officinale* methanol extract on endogenous antioxidant enzymes in $CuSO_4$-induced hippocampal neurotoxicity in mice. (A) GSH (B) CAT (C) SOD. Data are presented as mean±SEM (n=5), *p< 0.05, **p< 0.01, ****p< 0.0001 vs $CUSO_4$, and #p< 0.05, ##p< 0.01 vs control. GSH: reduced glutathione, CAT: Catalase, SOD; superoxide dismutase.

***Zingiber officinale* methanol extract inhibits pro-inflammatory cytokine release and neutrophil infiltration in $CuSO_4$-induced hippocampal neurotoxicity in mice**

Exposure to CuSO4 neurotoxicity increased the hippocampal inflammation expressed by elevated TNF-α [F (4, 10) = 15.12, p = 0.0003, fig. 4A], IL-1β [F (4, 10) = 47.77, p < 0.0001, fig. 4A] and MPO levels [F (4, 10) = 21.28, p < 0.0001, fig. 4C] comparative to control. However, MEZO treatment in dose-dependent effect (50 mg/kg) alleviated the hippocampal elevated pro-inflammatory cytokine release by reducing TNF-α and IL-1β levels compared to untreated CuSO4 exposed mice. However, treatment with MEZO (50 and 100 mg/kg) and Vit.C (100 mg/kg) alleviated the neutrophil infiltration in the hippocampus as evident with reduction in MPO level compared to untreated CuSO4 exposed mice.

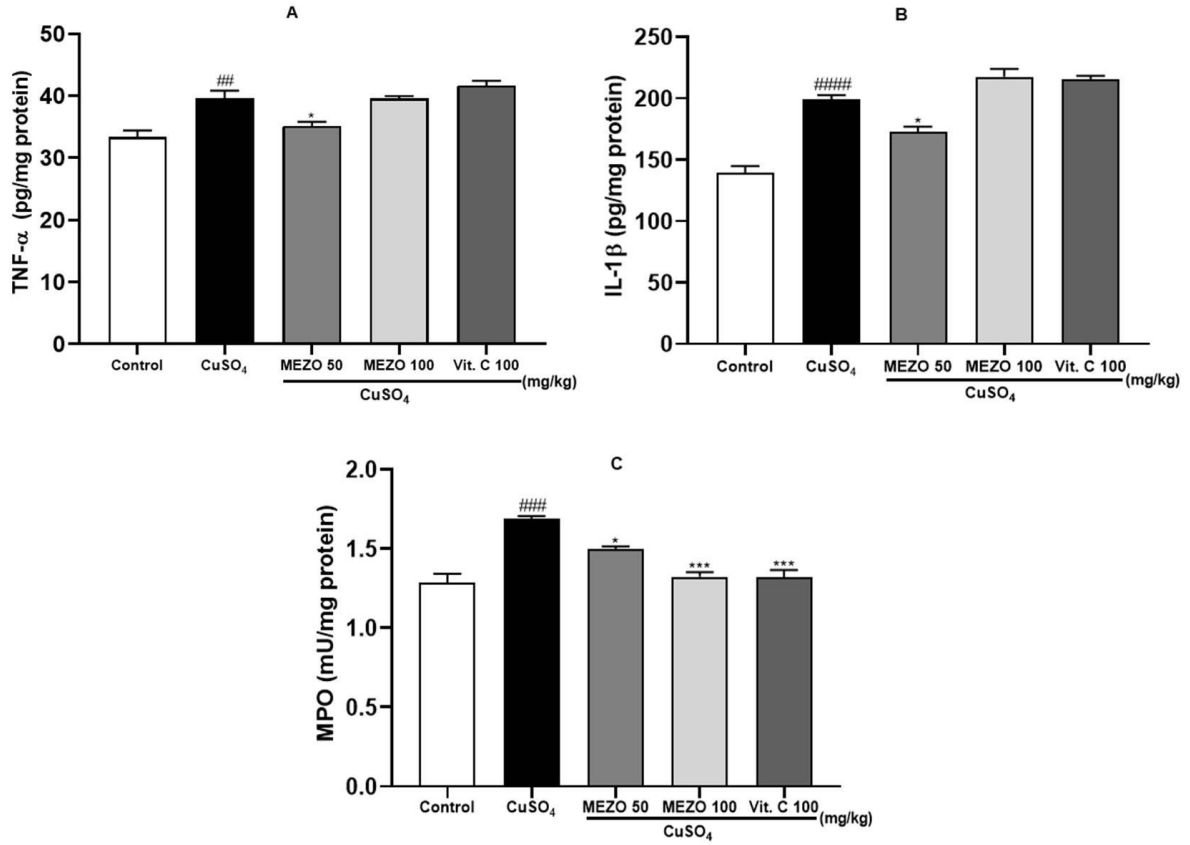


Figure 4 A-C: The levels of pro-inflammatory cytokine and neutrophil infiltration in $CuSO_4$-induced hippocampal neurotoxicity were reduced after *Zingiber officinale* methanol extract treatment in mice. (A) TNF-α (B) IL-1β, and (C) MPO. Data are presented as mean±SEM (n=5), *p< 0.05, ***p< 0.001 vs $CUSO_4$, and $^{\#\#}$p< 0.01, $^{\#\#\#}$p< 0.001, $^{\#\#\#\#}$p< 0.0001 vs control. TNF-α: Tumor necrosis factor-alpha, IL-1β: Interleukin 1 beta, MPO: Myeloperoxidase.

Zingiber officinale **methanol extract improves the release of Nrf2 protein and cholinergic transmission in $CuSO_4$-induced hippocampal neurotoxicity in mice**

As presented in fig. 5A and 5B, exposure to CuSO4 neurotoxicity significantly decreased the levels of Nrf2 [F (4, 10) = 34.05, p < 0.0001, fig. 5A] and increased the levels of AChE [F (4, 10) = 92.68, p < 0.0001, fig. 5B] contents in the hippocampus as compared with the control. Conversely, MEZO (50 and 100 mg/kg) and Vit.C (100 mg/kg) treatment significantly increased the Nfr2 and decreased the AChE contents in the hippocampus compared to untreated CuSO4 exposed mice.

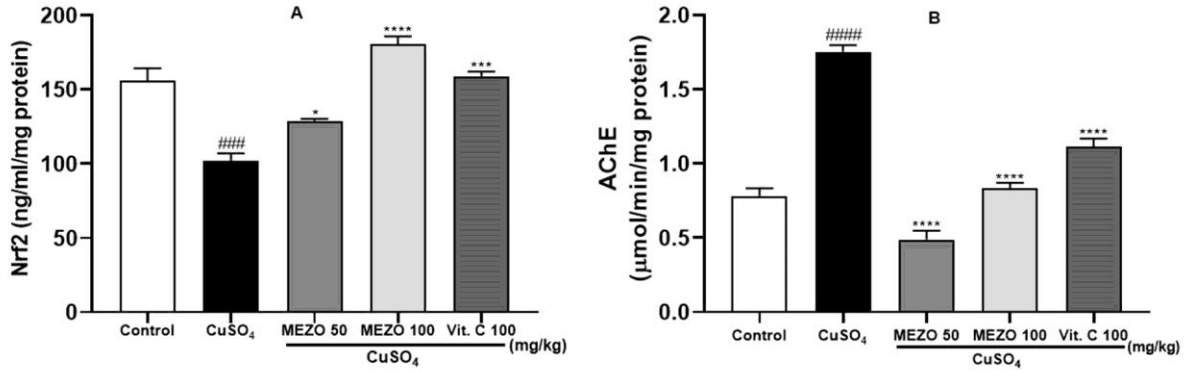


Figure 5 A-C: *Zingiber officinale* methanol extract improves the release of Nrf2 protein and cholinergic transmission in $CuSO_4$-induced hippocampal neurotoxicity in mice. (A) Nrf2, and (B) AChE. Data are presented as mean±SEM (n=5), *p< 0.05, ***p< 0.001, ****p< 0.0001 vs $CUSO_4$, and $^{\#\#\#}$p< 0.001, $^{\#\#\#\#}$p< 0.0001 vs control. Nrf2: Nuclear factor erythroid 2-related factor 2, AChE: Acetylcholinesterase.

Zingiber officinale **abates the degeneration of pyramidal neuron (CA3) in $CuSO_4$-induced hippocampal neurotoxicity in mice**

The photomicrograph and the result indicating the $CuSO_4$-induced hippocampal neurotoxicity effect in the mice brain is presented in fig. 6 and 7. $CuSO_4$ administration decreases the pyramidal neurons in the CA3 region significantly ($p < 0.05$) by eliciting atrophy, clumping and necrosis of the neuronal cells. However, treatment with MEZO (50 mg/kg and 100 mg/kg) and Vit. C 100 mg/kg significantly increased the survival of pyramidal neurons of the hippocampus in the mice [$F (4, 10) = 89.80$, $p < 0.0001$, Fig. 7] but with mild atrophic and necrotic neurons interspersed with several normal neurons in MEZO 100 mg/ kg and the Vit. C 100 mg/kg treated groups in comparison to the untreated CuSO4 exposed mice (Fig. 6 and 7).

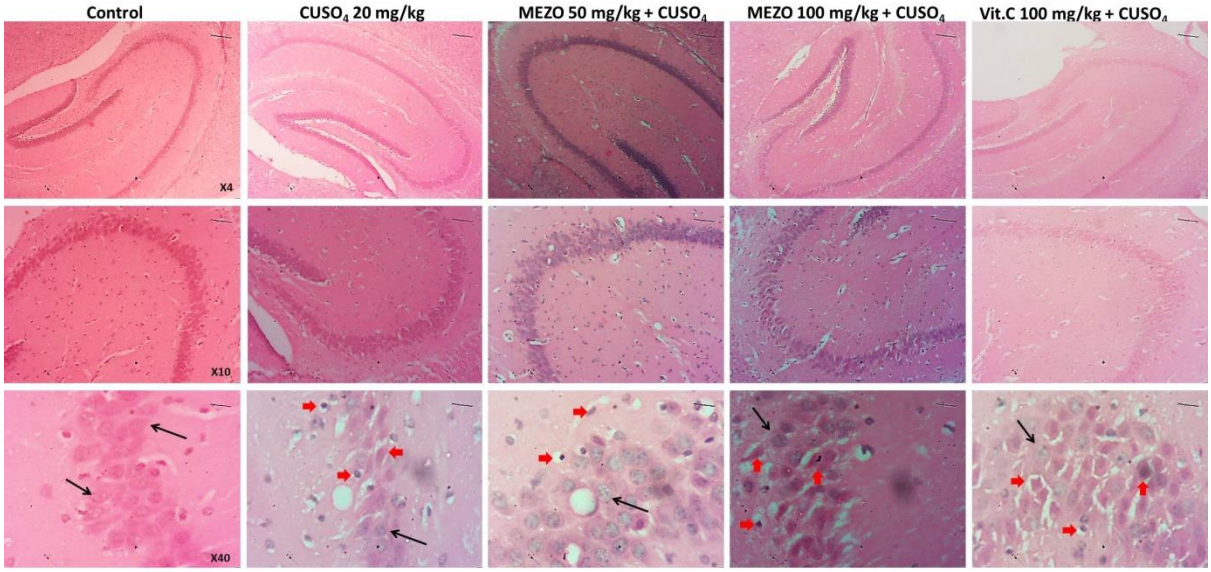
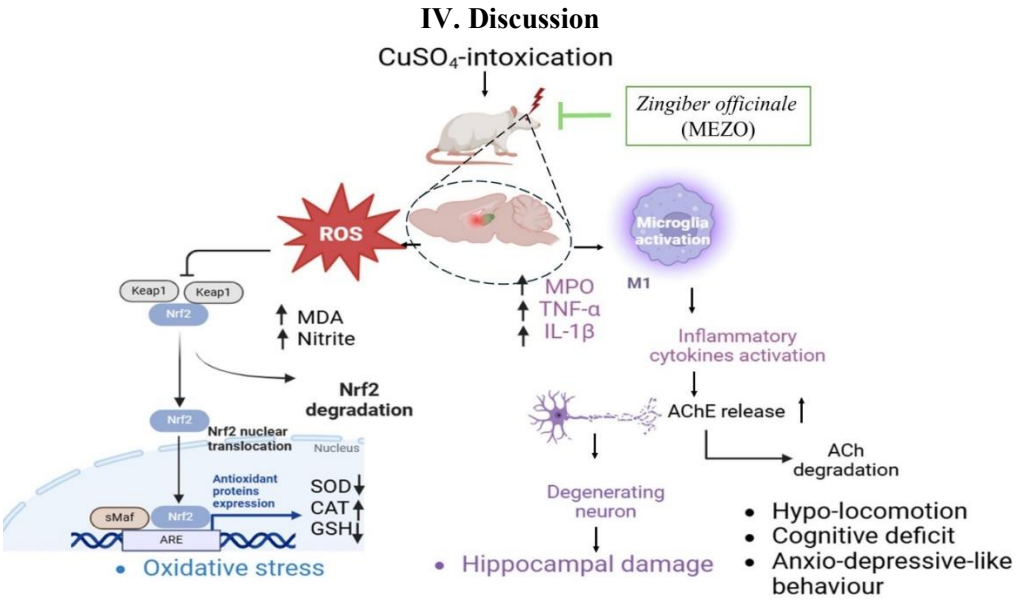


Figure 6 and 7: The photomicrograph representation of the effect of *Zingiber officinale* on CA3 neurons of the hippocampal subfield after hematoxylin and eosin staining in $CuSO_4$-induced hippocampal neurotoxicity in mice (Black arrow representing normal neuron; Red arrow representing degenerated neuron). Magnification = X100 (Fig. 6). The effect of *Zingiber officinale* on hippocampal neuron after hematoxylin and eosin staining in $CuSO_4$-induced hippocampal neurotoxicity in mice. Data are presented as mean±SEM (n=3), **p< 0.01, ***p< 0.001, ****p< 0.001 vs CUSO4; $^{\#\#\#\#}$p< 0.0001 vs control. (Fig. 7)



The result obtained from this study reveals the therapeutic role of *Zingiber officinale* Roscoe methanol extract (MEZO) in improving locomotion and cognition, and reducing anxiety- and depressive-like behavior, hippocampal redo-inflammation in copper II sulfate-induced neurotoxicity in mice. Cu exposure has been reportedly linked to hippocampal oxidative stress and neuronal degeneration[14,42] and recent studies have shown

that intraperitoneal injection of CuSO4 causes severe hippocampal neuronal death, impaired spatial learning and memory [14,43-44]. Similarly, CuSO4-supplemented drinking water at 10 mg/L which is above the daily recommended limit of exposure of 1.5–3.0 mg/L by the U.S. National Research Council[45] has been reported to cause elevated levels of acetylcholinesterase (AChE), malondialdehyde (MDA), β-amyloid precursor protein cleaving enzyme 1 (BACE1), phosphorylated Tau (p-tau), Caspase-9 (CAS-9), Bax, and TNF-α in rats hippocampus[46]. In corroboration with earlier studies[47,14,43,20,] the current findings revealed hypolocomotion in OFT, impaired spatial memory in Y-maze, and increased depressive-like and anxiety-like behavior in tail suspension test (TST) and light and dark transition test (LDT) in $CuSO_4$ intoxicated mice. Conversely, the MEZO co-treatment improved the motor performance indicated by in locomotor behavior, increased spatial memory function, reduced anxiogenic and depressive-like behavior in the mice.

Existing evidence suggests that disturbance in Cu metabolism increases the tissue susceptibility to oxidative damage[48-51]. In different tissues, Cu dysmetabolism have been reported to cause generation of reactive oxygen species such as hydroxyl (-OH) and superoxide (-O2) radicals which might damage biomolecules like DNA, proteins, and lipids thus heighten oxidative stress[52-53]. In different brain regions, elevated Cu level was reported to stimulate a Fenton-like reaction generating great amount of H_2O_2 via dismutase activity of SOD on superoxide radical and then CAT to catalyze the reduction of H_2O_2 to water[54]. This reaction confers the observed reduction in the activity of enzymatic and non-enzymatic antioxidant in perturbed brain Cu level. Our findings showed an elevated lipid peroxidation and nitrite levels and reduced GSH and SOD activity and increased CAT activity in hippocampal tissues of Cu-exposed mice suggesting oxidative stress. Few investigations have reported that marked inhibition of some antioxidant enzymes are associated with exposure to neurotoxicants, especially when given for several weeks and beyond the tolerable dose. However, the observed induction of enzymic antioxidant, precisely the GSH, CAT, and SOD after 28 days of $CuSO_4$ assault in this study evidenced an adaptive response of the hippocampal tissue to attempt to ward off the persisting $CuSO_4$ assault. Of note, the co-existence of the hippocampal adaptive response with damage to cellular biomolecules and consequence degeneration of the neurons is an indication of the progressive nature of $CuSO_4$ induced neurotoxicity, particularly at the first stage of the degeneration. However, our data demonstrated that MEZO mediated neuroprotection in the hippocampus by reversing the biochemical imbalance while preserving the hippocampal cells from oxidative damage and subsequent neuronal degeneration. Studies have identified GSH as a cofactor for glutathione related antioxidant system and detoxification enzymes including glutathione peroxidases (Gpx), glutaredoxins (Grx), glutathione S-transferases (GST), and glutathione reductase (GR) [55,53]. The hippocampal $CuSO_4$-lesioned mice demonstrated reduced glutathione level which suggests downregulation of the glutathione system. In accordance with the report of an earlier study who opines that large pool of glutathione is required to sustain hippocampal dendrite integrity and cognitive function, since slight decrease in glutathione level confer glial activation and dendrite dysfunction in the CA1 layer of the hippocampus[56], our data showed the MEZO treatment mitigated the CuSO4-induced lipid peroxidation and nitrergic activity, and enhanced the enzymatic glutathione system as well as the CAT and SOD system, respectively. Although, we hypothesized that the elevated hippocampal CAT activity may be due to the immune system triggered to combat the $CuSO_4$-induced biomolecule damage indicted by the increased level of MDA and nitrite. Interestingly, our finding confirmed MEZO antioxidant activity in the hippocampal tissue as earlier reported by several literatures to enhance memory and protect against brain damage in human and rodent [57-58.]

Further, our findings demonstrated that MEZO alleviated $CuSO_4$-induced hippocampal pro-inflammation cytokine release and neutrophil infiltration typified by a significant reduction in TNF-α, IL-1β and MPO levels. Note worthily, studies have shown that elevated inflammation in the hippocampal region impairs memory formation[59]. MPO activities, TNF-α and IL-1β are inflammatory and pro-inflammatory biomarkers implicated in the pathogenesis of neurodegenerative disease [60-61]. Targeting these biomarkers by inhibiting and reducing their activities could offer possible neuroprotective pathways to alleviate the progression of neurodegenerative disease. The result of this study showed that MEZO administration at 50 mg/kg effective inhibited TNF-α and IL-1β, while MEZO administration at 50 mg/kg and 100 mg/kg depleted the levels of neutrophil infiltration as indicated by reduced MPO activity in the hippocampus.

To substantiate our findings, we investigated the cytoprotective role of MEZO and its ability to enhance the antioxidant defense system through the Nrf2 pathway in CuSO4-induced hippocampal neurotoxicity in mice. Nrf2 regulates various expressions of gene that works to restore homeostatic activities and functions after oxidative stress and inflammation. More so, Nrf2 activation is essential to resist neuroinflammation, unfolded protein response, proteasome and autophagy, mitochondrial biogenesis and immune response[62-64]. Recent studies suggested strongly that Nrf2 played an important role in modulating neurocellular activities as well as becoming a strong therapeutic target for the treatment of neurodegenerative diseases precisely related to damage mitochondrial system[62-64]. Based on all the previously reported data, we therefore conclude that the Nrf2-mediated hippocampal neuronal protection against $CuSO_4$ assault is initiated by the hippocampal Nrf2 activation to modulate oxidative stress and inflammation. Our result showed the MEZO

upregulated the expression of Nrf2 protein in the hippocampus after $CuSO_4$ assault suggesting a probable mechanism through which $CuSO_4$-mediated hippocampal oxidative stress and inflammation were alleviated.

Alteration in the cholinergic synapses may contribute to the cognitive deficit while cognitive processes are improved when enhanced[65]. The above-mentioned neuropathobiologies have been supported by recent findings as $CuSO_4$ assault, in spite of its short-term administration, was observed to elicit significant alterations in the neuronal cholinergic system of the hippocampus, as depicted by elevated acetylcholinesterase release and decreased hippocampal production of endogenous antioxidant enzymes. Of note, this imply that short-term exposure to $CuSO_4$ assault induces damaging neurotoxicity to the hippocampal cells resulting to ACh depletion and AChE productions as recorded in the hippocampus of the assaulted mice. MEZO administration significantly improves ACh release evident by a decrease in AChE concentration in the terminals of hippocampal neurons and mitigated $CuSO_4$-assocaited cognitive and behavioral deficit. Hence, we suggest that MEZO administration is capable of potentiating or improving cholinergic transmission which could in turn enhance cognitive functions and behaviour.

From the histological evaluation of the hippocampus, we observed marked atrophy, clumping and necrotic neurons with vacuolated cytoplasm and condensation of the nuclei in the mice pyramidal neurons in the CA3 region of the hippocampus depicted by loss of viable cells. Interestingly, the treatment with MEZO abated the loss of histomorphological features of the mice pyramidal neurons in the CA3 region marked by viable cells, thus, elevating the viable neuronal cells population. Although, mild atrophic and necrotic neurons interspersed with several normal neurons were observed after MEZO treatment, the ability of MEZO to suppress the neuronal inflammation might be due the Nrf2 and endogenous antioxidant defense system activation, inflammatory inhibition, and AChE reduction in the hippocampus.

Taking together, our data showed that MEZO improves motor and memory functions, inhibits anxiety- and depressive-like behavior and AChE activity, depleted redo-inflammatory biomarkers by the upregulation of Nrf2 expression in $CuSO_4$ assaulted mice. We hypothesized that the protective effect of MEZO may be due to its antioxidant and anti-inflammatory properties. Our data provided evidence through which MEZO may be used as a therapeutic strategy against hippocampal degeneration following exposure to environment toxicants.

V. Conclusion

Taking together, our data showed that MEZO improves motor and memory functions, inhibits anxiety- and depressive-like behavior and AChE activity, depleted redo-inflammatory biomarkers by the upregulation of Nrf2 expression in $CuSO_4$ assaulted mice.

Availability of data: Data supporting this current study will be made available upon reasonable request from the corresponding author.

Conflicts of interest: Authors report no conflict of interest whatsoever

Funding: The work was funded by Tedfund through Institution Base Research (IBR) of Bayelsa Medical University (TETF/DR&D/CE/UPI/BAYELSA/IBR/2021)

Financial interest: Authors report no financial or non-financial interest whatsoever

CRediT authorship contribution statement:

AW: Conceptualisation, Methodology, Supervision, Formal analysis, Writing - review & editing. OGA: Methodology, Supervision, Formal analysis, Writing - original draft, Writing - review & editing. FAE: Methodology, Formal analysis. JC: Methodology, Writing - original draft, Writing – review & editing. MN: Writing - original draft, Writing – review & editing.

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