Wiley
Journal of Toxicology
Volume 2024, Article ID 8474801, 18 pages
https://doi.org/10.1155/2024/8474801

WILEY

Research Article

Spondias mombin Ameliorates Copper-Induced Memory Impairment in Mice by Mitigating Oxidative Stress and Modulating Cholinergic Activity

James Busayo Agboola,[1] **Amos Rotimi Joshua**,[1] **Sanmi Tunde Ogunsanya**,[2] **Adeola Folashade Ehigie**,[3] **Janet Olufumilayo Oke**,[4] **and Omodesola Oluwafisayo Ogunruku**[1]

[1]*Department of Biochemistry and Molecular Biology, Faculty of Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria*
[2]*Department of Anatomy, Benjamin S. Carson (Snr) College of Health and Medical Sciences, Babcock University, Ikenne, Ogun State, Nigeria*
[3]*Department of Biochemistry, Faculty of Basic Medical Sciences, Ladoke Akintola University of Technology, Ogbomosho, Nigeria*
[4]*Department of Cell Biology and Anatomy, Faculty of Basic Medical Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria*

Correspondence should be addressed to James Busayo Agboola; jagboola@pg-student.oauife.edu.ng

Received 4 June 2022; Revised 5 June 2024; Accepted 6 September 2024

Academic Editor: Zongming Ren

Copyright © 2024 James Busayo Agboola et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The study unveils the importance of *Spondias mombin* leaf extract (*S. mombin*: 100 mg/kg b.wt.) in mitigating the toxic insult mediated by copper (16 mg/kg) on the cholinergic system via oral gavage. Male mice were weighed and distributed into five groups, six mice per group. Group A represents the basal control group given only distilled water; Group B and C were given 16 mg/kg of copper sulphate and 100 mg/kg of *S. mombin* methanol leaf extract, respectively, for 31 days; Group D was given 16 mg/kg of copper sulphate for 31 days, followed by treatment with 100 mg/kg of *S. mombin* methanol leaf extract for 15 days; and Group E was assigned only *S. mombin*. The copper concentration was evaluated using atomic absorption spectroscopy, while the cognitive abilities were assessed using Morris water maze and elevated plus-maze. The result showed that *S. mombin* reversed the disparities in cholinesterase and choline acetyltransferase activities in vivo due to copper neurotoxicity, with a relative ($p < 0.05$) decrease in brain and blood copper concentration in co- and posttreatment groups. Furthermore, *S. mombin* reported decreased the disparity between pro- and antioxidant occurrence caused by copper toxicity by lowering brain lipid peroxidation, raising thiol levels, and increasing the activities of antioxidant enzymes such as glutathione transferase, catalase, and superoxide dismutase. The result showed that the extract had a protective influence on memory deficits ($p < 0.05$), significantly reduced anxiety levels, and increased the overall number of neurons in the hippocampus subfields. In conclusion, *S. mombin* ameliorates Cu-induced neurotoxicity and neurocognitive dysfunctions by virtue of enhancement of cholinergic signalling and antioxidant activities in the brain.

Keywords: anxiety; choline acetyltransferase; cholinesterase; memory impairment; neurotoxicity; oxidative stress; *Spondias mombin*

1. Introduction

Copper (Cu) exposure is a major environmental health problem [1, 2]. Although it is the third most essential micronutrient element in the human body [3], global copper production is steadily increasing, and its incidents of exposure have been reported via the water supply, occupational chemical hazards, use of herbicides and pesticides, environmental emissions, industrial waste and the use of Cu-containing contraceptive implants [1, 2, 4]. Copper can

be absorbed from copper plumbing pipes giving rise to elevated copper levels (exceeding 100 mg/L) in drinking water [5]. The Scientific Committee on Food (SCF) concludes that no retention of copper is expected to occur with an intake of 5 mg/day and established an acceptable daily intake (ADI) of 0.07 mg/kg bw [6, 7]. Within the framework of evaluating plant protection products (PPPs), EFSA established an ADI of 0.15 mg Cu/kg bw per day in 2008 [6, 7]. This is equivalent to 10 mg per day for an adult weighing 70 kg. EFSA validated this ADI value in 2018 as part of the peer review process for renewing the licencing of copper as a PPP. EFSA developed appropriate intakes (AIs) in 2015 to prevent copper deficiency in the context of copper as an important nutrient [6, 7].

The central nervous system has been identified as the primary target for Cu-induced toxicity, with ongoing debates on the involvement of copper dysfunctions in neurological conditions. Environmental copper exposure, for example, is a hallmark of many neurodegenerative disorders (NDs) and cognitive disability impairment [8, 9]. Its neurotoxic mode of action seems to have a direct or indirect role in elevated oxidative stress, a significant factor in neuronal toxicity [10]. Although there is still much more to discover about Cu neurotoxicity in neurodegenerative and its involvement in disease aetiology [11], therapeutic strategies to regulate Cu levels and interactions with Cu-binding proteins in the central nervous system are currently under investigation [11, 12]. With the upward rise in NDs, it is critical to identify and understand the processes by which copper exposure could negatively affect cognitive function and increase the risk [13]. This investigation therefore employed copper as a neurotoxicity-induced model and its connection with cognitive performance and how these could be aimed for therapeutic intervention.

Diverse mechanistic processes play a role in Cu-induced neurotoxicity, and the mechanism underlining its cognitive function is controversial and not well understood. However, the most common mechanism underlining Cu-induced toxicity was attributed to the Fenton reaction [14, 15], a reaction that eventually generates brain-reactive oxygen species (ROS) and hence neuronal cell damage [14, 15]. In addition to this pathway, Cu has the ability to react with hydrogen peroxide to form hydroxyl radical [15, 16], a reactionary radical that can damage macromolecules such as membrane proteins, lipids, DNA and RNA [15]. Although Cu has been shown to cause several biochemical cascades through oxidative stress that significantly results in brain inflammatory responses and neuronal death [8, 9, 17], the contribution of oxidative stress to increased acetylcholinesterase (AChE) activity has been identified in the pathologies of NDs as well. For instance, AChE degrades acetylcholine (ACH), a presynaptic modulator [18–21], and it is widely assumed that ACH plays a pivotal role in learning and memory processing. Its deficiencies have been linked to higher cognitive impairment as seen in NDs [22, 23]. Therefore, brain ACHE inhibition could be a primary therapeutic target in NDs treatment strategies, by strengthening the level and duration of neurotransmitter action [18, 24].

Medicinal plants are abundant in exogenous antioxidant defences such as flavonoids and carotenoids and have the potential to safeguard cognitive function [21, 23, 24]. Prior research has shown that medicinal plants could modulate the levels of antioxidant indicators, such as SOD, catalase (CAT) and glutathione, therefore enhancing spatial learning and memory. On the other hand, it has been shown that the consumption of food plants has the potential to inhibit ACHE, thereby leading to the restoration of cognitive function in animals [24]. Therefore, we further evaluated the amelioration potentials of *S mombin* in Cu-induced perturbations. *S mombin*, also called iyeye in Yoruba ethnomedicine [25, 26], has been utilized by Nigerian traditional practitioners to treat a variety of nerve disorders. The outcomes from high-performance liquid chromatography-ultraviolet fingerprint analysis shows the presence of flavonoids phytoconstituents, as well as the identification of ellagic acid and isoquercitrin, whereas the vast bulk of the compounds by the gas-chromatographic technique seemed to be fatty acids, esters and alcohols. The fruit, leaf or bark of *S. mombin* has been shown to have antidiarrheal, antibacterial, diuretic, febrifuge and emetic properties. The flower, leaf or bark are used to treat stomachaches and other inflammatory diseases, as well as to heal wounds. The leaf and fruit have been shown to have antioxidant properties [27]. Its leaves have anti-inflammatory, anthelmintic, haematinic and sedative properties, and its stem bark has antimycobacterial properties [25, 26]. Phytochemical screening revealed that *S. mombin* leaf (SML) contains tannins, saponins, alkaloids, flavonoids and phenols in a previous study [28]. It also contains riboflavin and thiamine, as well as ascorbic acid and niacin. Despite the leaf's information, little is known regarding its scientific utility in the CNS. Therefore, the study aims to report the potentially beneficial effects of *S. mombin* on copper-induced neurotoxicity.

2. Materials and Methods

2.1. Botanic Material and Extract Preparation. Fresh *S. mombin* leaves were collected from the Botanic Garden of Obafemi Awolowo University in Ile-Ife, Nigeria. A sample of the collected plant material was identified and deposited in the Ife Herbarium, Department of Botany, Obafemi Awolowo University, under the exsiccate number IFE17618. Weighed fresh leaves (300 g) were air-dried and powdered. The powdered leaves were then extracted using 80% of methanol with 20% distilled, with constant shaking, and thereafter filtrated. The solvent was filtered through the funnel, making use of cheesecloth and fluted filter paper. The filtrate was concentrated using a rotary evaporator and thereafter stored at 4—8 C until further use.

2.2. Phytochemical Investigation. The phytochemical constituents were identified using necessary preliminary phytochemical screening as described by the authors of [29–31].

2.3. Estimation of Total Phenolic Content. In this study, we employed the use of the Folin–Ciocalteu method to evaluate the total phenolic content of *Spondia mombin* [32]. In

a nutshell, 0.1 mL of *S. mombin* extract (1 mg/mL) and 0.1 mL of Folin–Ciocalteu phenol were mixed and waited for 5 min. Following that, 1 mL of 7% Na_2CO_3 and 1.3 mL of distilled water were introduced. The reaction mixture was kept at 29°C for 90 min. At 750 nm, maximum absorbance (OD) was measured. The total phenolic value was observed as gallic acid equivalent (mg GAE) per gram of dry leaf extracts using the calibration graph of reference gallic acid solutions. All parameters were measured three times.

2.4. Determination of Total Flavonoid. The actual flavonoid content was estimated using Meda et al. [32], aluminium chloride approach. To begin, 0.3 mL of *S. mombin* (1 mg/mL), 0.15 mL of AlCl.6H O (0.3 M), 0.15 mL of $NaNO_2$ (0.5 M) and 3.4 mL of methanol (30%) were dispersed together. The reactivity was terminated after 5 min with 1 mL of NaOH (1 M). At 506 nm, the optical density was taken. The total flavonoid concentration was evaluated as quercetin equivalent (mg QE) approximation per gram dry plant extract using a curve of conventional quercetin solutions and demonstrated as mg QE per gram dry crude extracts. All samples' data were measured in triplicate.

2.5. Experimental Design

2.5.1. Obtaining Copper Sulphate. Copper sulphate grade of 95% purity was obtained from Sigma-Aldrich, Inc. (St Louis, MO, USA).

2.5.2. Experimental Animals. Thirty male Swiss albino mice (*Mus musculus*) weighing approximately 22–25 g were acquired from the Pharmacy Faculty's Animal House at Obafemi Awolowo University in Osun State, Nigeria. Before the experimental study, all the mice were maintained at an optimal temperature range of 65–80 degrees Fahrenheit and a humidity level between 40% and 70%. The animals were kept for 2 weeks under the light and dark cycle. All animals were supplied with a standard chow diet and had unlimited access to fresh water. Ethical approval for this research was obtained from the Health Research and Ethics Committee of the Institute of Public Health, Obafemi Awolowo University (HREC No: IPH/OAU/12/2050).

2.6. Experimental Grouping. A total of 30 male mice were weighed and distributed into five groups, six mice per group (Scheme 1).

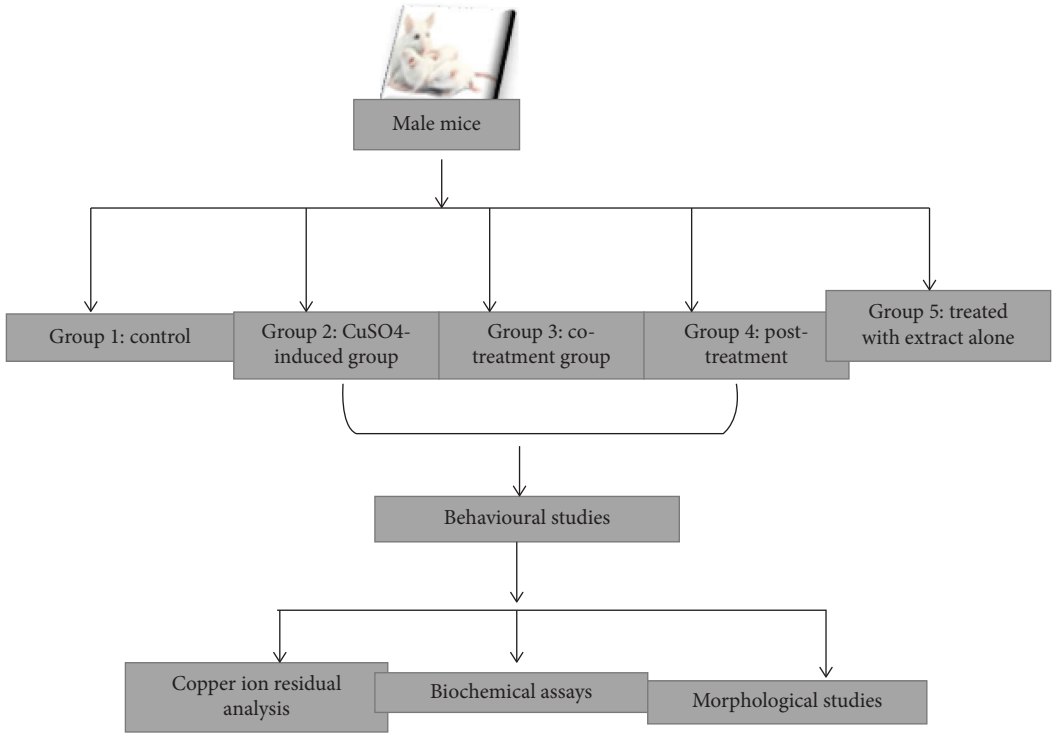
- Group A: The control group treated only with distilled water.
- Group B: It was treated with 16 mg/kg copper sulphate.
- Group C: It was administered with 16 mg/kg of copper sulphate and cotreated with 100 mg/kg of *S. mombin* leave extract for 31 days.
- Group D: It was administered with 16 mg/kg copper sulphate for 31 days, followed by the treatment with 100 mg/kg *S. mombin* for 15 days.
- Group E: It was administered only with 100 mg/kg of *S. mombin* leaves extract for 1 month.

Copper sulphate was dissolved in distilled water, and the $CuSO_4$-treated groups received intragastric doses of 16 mg/kg of $CuSO_4$ as reported by Liu et al. [33]. Related dose was also reported by Kumar, Sathua and Flora [34] to induce neurotoxic effects in rat brain (Schemes 1 and 2). The mice were administered through the oral gavage (1 mL) with 100 mg/kg of the extract as reported by Ola-Davies et al. [35] and Brito et al. [36]. Group C was placed on 100 mg/kg of *S. mombin* 2 h before receiving 16 mg/kg intragastric $CuSO_4$ dosage for 31 days. Group D was placed on 16 mg/kg of $CuSO_4$ continuously for 31 days after which it was treated with 100 mg/kg *S. mombin* for 15 days.

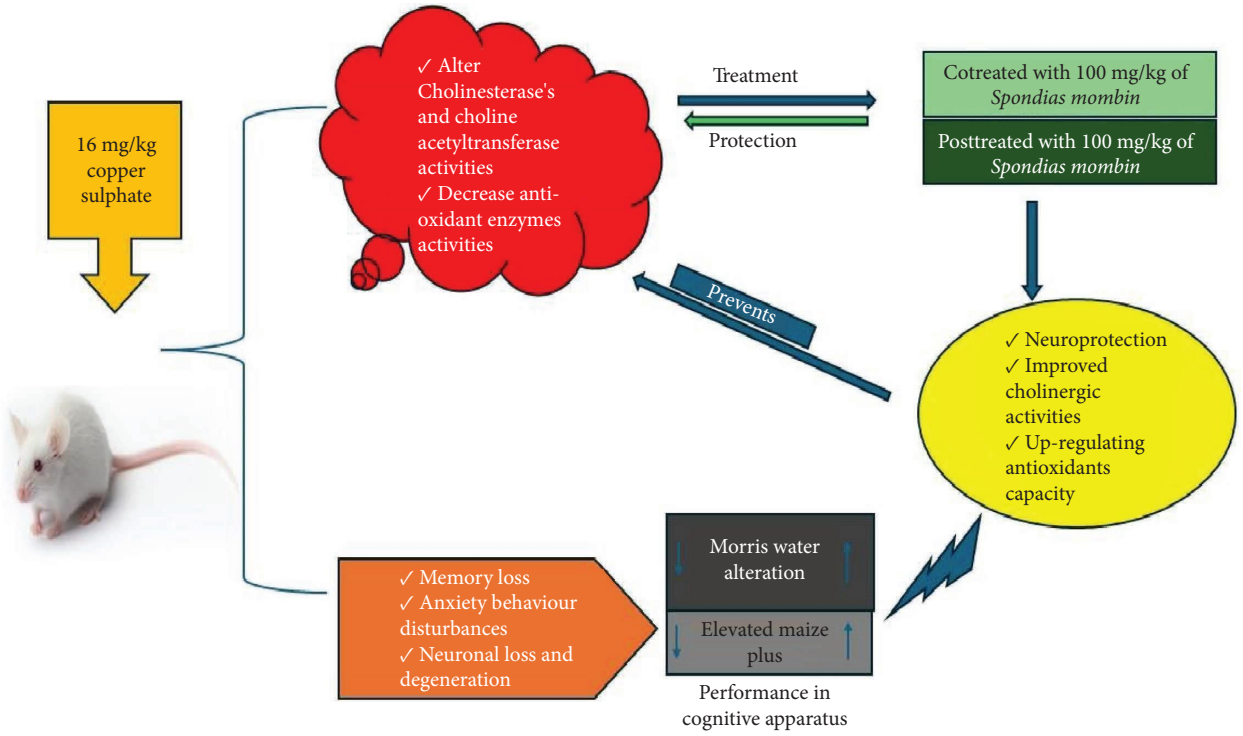
2.7. Behavioural Tests. Thirty minutes before the behavioural activity, the mice cages were moved from the animal house to a behavioural room in the morning (Scheme 1). The animals were examined using the Morris water maze (MWM) and elevated plus-maze (EPM) test paradigms to establish baseline cognitive abilities as well as anxiety-like behavioural activity (before treatment with the plant extracts). On day 31, these steps were repeated (i.e., the last day of both co- and posttreatment with the extract). The behavioural study was conducted during the day using a reversed day/night cycle. It was carried out during the daylight in a dark room under a red-light condition [37]. The EPM test was conducted between 7:45 a.m. and 10:15 p.m. while Morris's water test was conducted 4 h later. The walls and floors of the elevated plus-maze apparatus were cleansed with 10% ethanol solution after each test to remove any smell cues.

2.8. EPM Test. The anxiety-like activity was assessed using the EPM [38]. The EPM is a commonly used paradigm to assess anxiety-related anomalies in mice and rat models. It is a construction made of a greyish polyvinyl chloride sheet with two equal open arms (measuring 50 cm by 12 cm) and two closed arms (measuring 50 cm by 12 cm). The central square is about 12 cm by 12 cm, providing a pathway for the mice to traverse between the arms. The labyrinth's open arms had a 0.5 cm wall to prevent animals from falling over, and the maze was situated about 50 cm above the floor. A video camera was used to automatically assess physical indicators, such as the duration of time spent in the open arms and the frequency of movements between the closed and open arms. Once all four paws passed into arms, the entry was taken into account. The experiments were videotaped and analysed using the duration spent travelling to the EPM arms. We use an experienced visual clue in the other interpretative analysis.

2.9. MWM Task (MWM). The MWM task was used to assess learning and memory activities [39]. The screening arena was a round pool filled with water (100 cm in diameter and 40 cm in height), with an invisible platform (10 cm in diameter and 20 cm in height) immersed in water just below



Scheme 1: A diagrammatic sketch for experimental design.



Scheme 2: Protective mechanism of *Spondias mombin* on Cu^{2+}-induced toxicity in male mice.

the water's surface. The water's depth was 15.5 cm, and it was opaque. During the probe test, the platform was removed, allowing each mouse to swim openly for 120 s. The mice learn to escape from the water by finding the platform, which is usually done with the assistance of visual cues. The major purpose of these tests was to see how quickly the mice could locate the platform and how this ability improved over time. Furthermore, the platform was removed in the probe trial, and the mice were permitted to look for the omitted platform for 60 s. The time spent by the mice in the target

quadrants was recorded, and this was thought to signify the degree of memory consolidation. The rate of platform crossings as well as the average duration spent in the target quadrant was documented.

2.10. Brain Preparation. After the behavioural assessments, the animals were sacrificed by cervical dislocation. Before cervical dislocation, the animals were euthanized using a diluted solution of sodium pentobarbitone administered intraperitoneally. The anaesthetics dosage of 40 mg/kg was given intraperitoneally to the mice. The brain was extracted, and the animals were confirmed dead in a humanely killed manner before disposal. This study makes use of the whole brain homogenate. The brain was sectioned vertically into the right and left lobes. One section was stored inside the refrigerator at −20 C for copper determination using atomic absorption spectrophotometry (AAnalyst 400 Perkin Elmer), while the other section was put on ice and rinsed with ice-cold isotonic saline. The tissues were homogenized in 10 volumes of 0.1 M phosphate-buffered saline (pH 7.4). The homogenate was thereafter centrifuged at 4000 rpm for 15 min, and the supernatant was collected and stored at −20 C for subsequent biochemical assays.

2.11. Copper Ion Residual Analysis. Brain and blood copper concentration was determined by using atomic absorption spectrophotometry (AAnalyst 400 Perkin Elmer) at the central science laboratory, Obafemi Awolowo University, Ile-Ife, Nigeria. Blood was collected through the cardiac puncture, while 0.8 g of brain tissue was placed in a screw-capped tube and dried properly in the oven at 45 C. The dried tissue samples were then ground into a fine powder and thereafter transferred to a Teflon beaker. 20 mL of freshly prepared aqua regia (HCl: HNO_3 (3:1)) was added, and this was heated to near dryness; heating was removed and allowed to cool to room temperature; 20 mL distilled water was then added and heated again for about 3–5 min; this was then allowed to cool and filtered. The filtrate was made up to mark in a 25 mL flask and kept refrigerated pending metal determination by atomic absorption spectrometer (AAS).

2.12. Hippocampus Tissue Processing. Two mice per group were picked randomly for histopathology studies (Scheme 1). The brain was collected and prerinsed with a mixture containing 0.9% NaCl and 1 M ethylenediaminetetraacetic (EDTA) acid, followed by 2 h of 4% paraformaldehyde. The cranial nerve was then severed, separating the brain from the cranium. The brain tissue samples were fixed in 4% formalin, engrained in paraffin, and microtome-sliced into 20 mm thick slices. Thereafter, the slides were stained with haematoxylin and eosin before being inspected under a light microscope. The structure of the hippocampal area was noted under a light microscope at 400 magnifications. The number of pyramidal neurons in the hippocampal region was estimated. Image J cell counter tool was used to identify and quantify the number of intact and degenerating neurons in H&E-stained sections. For evaluation of neuronal count, the grid was superimposed on the photomicrograph with an area per point of 4000 μm^2. To quantify neuronal degeneration, the following morphological criteria were considered: cell swelling and lysis, pyknosis, eosinophilic neurons and vacuolated neurons [40]. A photomicrograph of ×400 magnification was used for this research.

2.13. Protein Concentration. Protein concentration was determined in the cerebral cortex according to the method of Bradford [41], using bovine serum albumin (BSA) as the standard, and the protein concentration in each homogenate was extrapolated from the standard curve. The reaction mixture contained 100 μL of protein source diluted in five volumes of distilled water and 1000 μL of Coomassie brilliant blue G-250 (Bradford reagent). At 595 nm, the absorbance was measured. The assay's concept is focused on the capacity of Coomassie brilliant blue G-250 (a dye) to interact with amino acids (arginine, lysine and histidine) in proteins under acidic conditions, shifting the dye absorbance from 465 nm to 595 nm.

2.14. Biochemical Guided Assays

2.14.1. AChE and Butyrylcholinesterase (BuChE) Assay (In Vivo). The AChE was assessed using the Ellman et al. [42]. The approach relies on thiocholine generated during the enzymatic hydrolysis from acetylthiocholine and reacts with Ellman's reagent to produce yellow 5-thio-2-nitrobenzoate. The reaction mixtures of 200 μL of supernatant (0.342—0.435 mg of protein), 100 μL of 3.3 mM 5, 5″ -dithiol-bis (2-nitrobenzoic) acid (DNTB) and 500 μL of phosphate buffer (pH 8.0) were incubated at 25°C for 20 min. Acetylthiocholine iodide (100 μL) was added to the mixture to initiate the reaction. The absorbance at 412 nm was measured every 15 s for 3 min.

Butyrylcholine iodide was used as a substrate to measure BuChE enzyme activity as also described by Ellman et al. [42]. Specific activity = $(\Delta abs \times Av \times Df)/(\in(DTNB) \times Ev \times Pc \times L)$ where Δabs, Av, Df, Ev, Pc and L represent a change in absorbance, assay volume, dilution factor, enzyme volume, protein concentration and lightpath, respectively.

2.14.2. Choline Acetyltransferase Bioassay. We carried out choline acetyltransferase activity by adopting the method of Fonnun [43], using [3H]AcCoA as substrate. The medium contains 384 mM NaCl, 10 mM choline chloride, 25 mM Na-EDTA, 10 mM eserine sulphate and 12 MAcCoA. At every 15 s, at 37°C, for 5 min, 20 L of [3H]AcCoA (0.1 Ci) and 20 g of protein were added to the assay tube with 10 L of the substrate solution. The process was halted by adding 50 mM Na-EDTA along with 2 mL of Kalignost solution (5 mg/mL tetraphenylborate in acetonitrile). The activity is reported as moles of [3H]ACh produced per minute per mg of protein and was determined using a liquid scintillation Packard apparatus (Model 1600 TR). The assay was in triplicate.

2.14.3. Lipid Peroxidation Assay (Thiobarbituric Acid Reaction). This study makes use of the Ohkawa et al. method. [44], to measure the level of lipid peroxidation. The reaction mixture contained 100 μL of homogenate and 30 μL of 0.1 M tris HCl buffer (pH 7.4) and was incubated at room temperature oC for 1 h before adding 300 μL of 8.1% sodium dodecyl sulphate, 500 μL acetic acid/HCl (pH 3.4) and 500 μL% TBA (thiobarbituric acid). The reaction mixture was incubated at 100 C for 1 h to promote colour development. At 532 nm, the absorbance of thiobarbituric acid reactive (TBAR) species produced was measured. The level of malondialdehyde (MDA) was then determined by calculating mol/mg protein ($=1056 \times 105\ M^{-1}{\cdot}cm^{-1}$).

2.14.4. Total Thiol Concentrations. The total thiol level in the cerebral cortex was determined using the method of Ellman et al. [45]. The reaction mixture contained 270 μL of 0.1 M of potassium phosphate buffer (pH 7.4), 20 μL of prepared supernatant and 10 μL of 10 mM (5,5-dithiol-bis-(2-nitrobenzoic acid)) (DTNB). Following incubation for 30 min at room temperature, the absorbance was measured in a spectrophotometer. These methods depend on the oxidation of DTNB to 5-thio-2-nitrobenzoic acid (TNB), which is spectrophotometrically active at 412 nm. The total thiols values were expressed in μmol/mg protein.

2.14.5. Glutathione-S-Transferase (GST) Activity. The activity of GST was measured using Habig and Jakoby's [46] method using 1-chloro-2, 4-dinitrobenzene (CDNB) as the substrate, 263 μL sodium phosphate buffer (0.1 M, pH 7.0 with 2.5 mM EDTA): 10 μL homogenate and 20 mM GSH (15 L): 25 mM CDNB (12 L) At 340 nm, absorbance increased for 3 min at 10 s intervals. The extinction coefficient of $9.6\ mM^{-1}{\cdot}cm^{-1}$ for the coloured GS–DNB conjugate formed by GST was used to calculate the data in mol/min/mg of protein.

2.14.6. CAT Activity Assay. CAT activity was determined using the procedure described by Aebi [47]. Briefly, 50 μL of the prepared supernatant was added to a cuvette containing 500 μL of 0.1 M phosphate buffer (pH 7.4) and 500 μL of 20 mM H_2O_2. The capacity of CAT to degrade H_2O_2 into H_2O and O_2 is required for the dominant reaction. Change in absorbance was read at 240 nm for 1 min using a spectrophotometer. The molar extinction coefficient of H_2O_2 $43.6\ M{\cdot}cm^{-1}$ was used to determine the CAT activity. One unit of activity is equal to 1 mmol of H_2O_2 degraded per minute and is expressed as units per milligram of protein.

2.14.7. Superoxide Dismutase (SOD) Activity. The activity of SOD was ascertained using McCord and Fridovich's method, [48]. To 20 μL of prepared supernatant, 75 mM Tris-HCl buffer (pH 8.2) comprising 30 nM EDTA and 30 μL of 2 mM pyrogallol were added. SOD activity is commonly measured using implicit assays of superoxide consumption, such as pyrogallol autoxidation. A spectrophotometer was used to measure the transformation in absorbance at 420 nm for 3 min at 30-s durations. The rate of autoxidation of pyrogallol is 50% inhibited by one unit of enzyme activity, as measured by the change in absorbance/min at 420 nm.

2.15. Statistical Analysis. Graph Pad® Instant Statistical Package Prism 5 was used for statistical analysis. The results were pooled ($n = 6$) and expressed as mean and standard deviation (means SD). The means were analysed using one-way (for biochemical analysis) and two-way analysis of variance (for behavioural analysis), accompanied by Turkey's multiple comparison test. For all comparisons, p values of 0.05 were considered significant.

3. Results

3.1. Preliminary Phytochemical Composition of Methanolic Leaf Extract of S. mombin. The extract reveals the presence of tannins, anthraquinones, flavonoids, cardiac glycosides and saponins, concerning phytoconstituents analyses. However, the extracts lacked phlorotannins and alkaloids, and in addition to this, we established that the methanolic extract of *S. mombin* contains phenol.

3.2. Standardization of the Extract's Phytoconstituents and Bioactive Properties. The total phenolic deductive approach of *S. mombin* extract revealed 127.34 ± 4.34 mg of total phenolic content (GAE) per gram of dried extract as well as 8.79 ± 0.47 mg of total flavonoid (quercetin equivalent) per gram of dried extract.

3.3. Effect of Methanol Extract From S. mombin on Behavioural Activities in Mice Exposed to Copper Sulphate

3.3.1. Amelioration of Learning and Memory Deficit by S. mombin in Mice Exposed to Cu-SO_4. Figures 1(a), 1(b), 1(c) and 1(d) present the effect of *S. mombin* on learning and memory deficit in the mice exposed to copper sulphate. Both co- and posttreatment trials show cognitive deficit ($p < 0.05$) due to copper neurotoxicity as observed in the Morris water paradigm as shown in Figure 1 and Table 1; that is, the copper-induced groups in both phases spent less time in the target quadrants, and there was increased escape latency due to copper toxicity compared to the control group. Surprisingly, in both the co- and posttreatment trials, *S. mombin* extracts at 100 mg/kg abrogated the cognitive deficit ($p < 0.05$) observed in this study due to copper neurotoxicity. This is typified by the significant decrease in escape latency ($p < 0.05$) and increase in the time spent in the target quadrants (Table 1) after 30 and 15 days of co- and posttreatment with the extract, respectively.

3.3.2. Effect of the Extract on Anxiety-Like Behavioural Activities. As represented in Figures 2(a), 2(b), 2(c) and 2(d), the baseline measurements carried out before treatment with the plant extracts in both co- and posttreatment trials show an increase in anxiety-like behavioural disturbances ($p < 0.05$) for all the groups exposed to copper when compared with the control; that is, this was depicted by a decrease in the number of entries ($p < 0.05$) in the close-

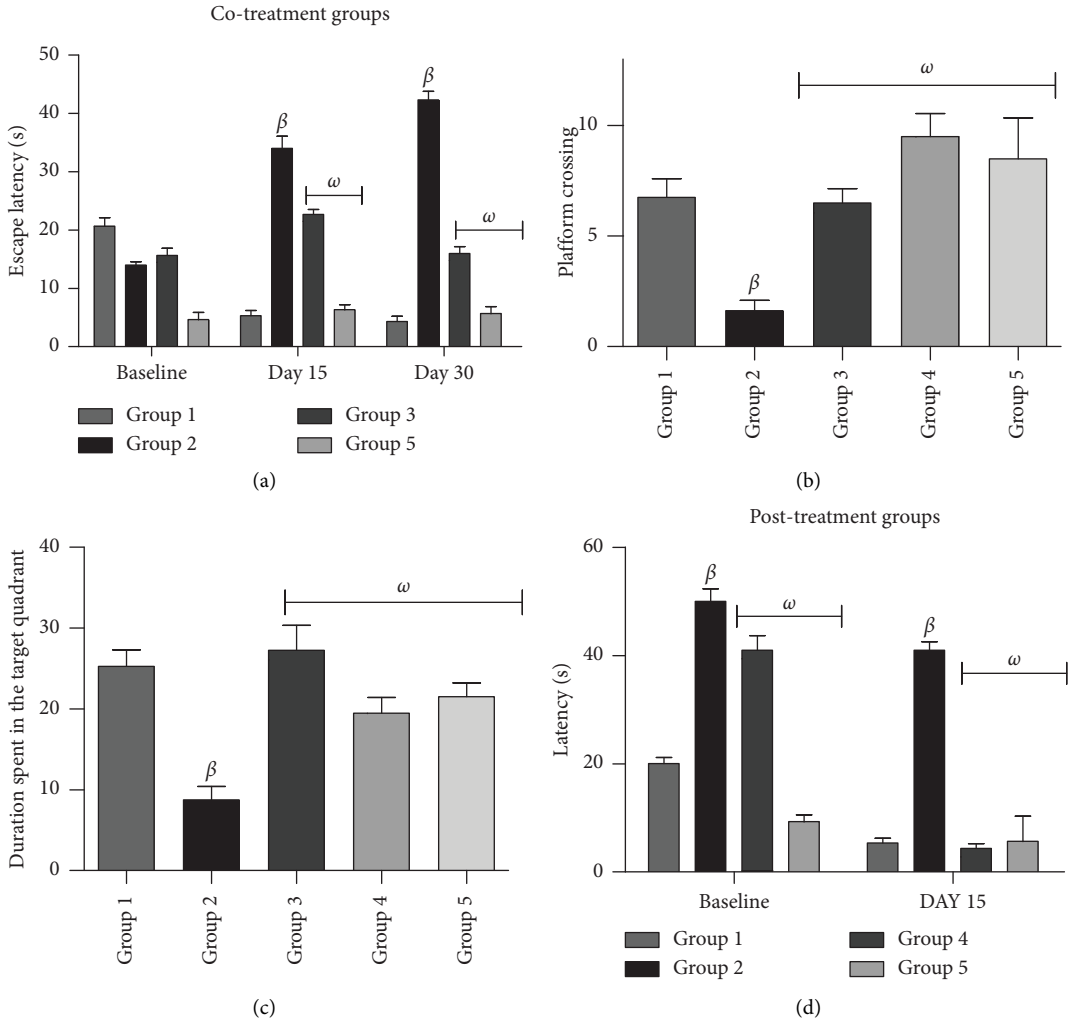


Figure 1: Effect of *S. mombin* on learning and memory acquisition after copper-induced neurotoxicity using Morri's water maze test. (a and d) The escape latency in co- and posttreatment trials. (b) The amount of time spent in the target quadrant after the platform was removed in both trials. (c) Platform crossing was used to determine the action on the spatial memory. Given that the statistical data were expressed as mean ± SD ($n = 6$). β represents a significant difference between the Cu-induced group (group 2, i.e., without treatment) and the control, ω represents a significant ($p < 0.05$) difference between the treated groups (groups 3 and 4) and Cu-induced group. Group 1: (control); group 2: ($CuSO_4$ group only); group 3: (cotreatment group); group 4: (posttreatment group); group 5: (treated with extract alone).

Table 1: Time spent in each quadrant target when compared to the control group and $CuSO_4$ group.

Groups	First quadrant	Second quadrant	Third quadrant	Target quadrant
Group 1	15.04	16.75	17.23	25.23
Group 2	10.45	9.65	27.32	10.36^{β}
Group 3	19.67	18.43	20.54	27.18^{ω}
Group 4	25.18	26.28	18.21	25.45^{ω}
Group 5	22.25	20.14	16.09	29.54

Note: group 1: (control); group 2: ($CuSO_4$ group only); group 3: (cotreatment group); group 4: (posttreatment group); group 5: (given only the extract alone). β represents a significant difference between the Cu-induced group (group 2, i.e., without treatment) and the control, and ω represents a significant (P < 0.05) difference between the treated groups (groups 3 and 4) and the Cu-induced group. Group 1: control; group 2: CuSO4 group only; group 3: cotreatment group; group 4: posttreatment group; group 5: treated with extract alone.

arm and an increase in the time spent in the open arms in elevated maize plus. This indicated that mice under anxiety-like behavioural activity had a lower tendency to stay in an open arm in proportion to the control group. On the contrary, when compared to the copper-induced groups, co- and posttreatment trials treated with *S. mombin* on Days 30 and 15, respectively, abrogate this effect ($p < 0.05$). This is an indication of decreased behavioural disturbances. That is, this confirmed a significant increase ($p < 0.05$) in the number of entries (Figures 2(a), 2(b), 2(c) and 2(d)) and the time spent in the open arm after treatment with *S. mombin* extract in co- and posttreatment trials.

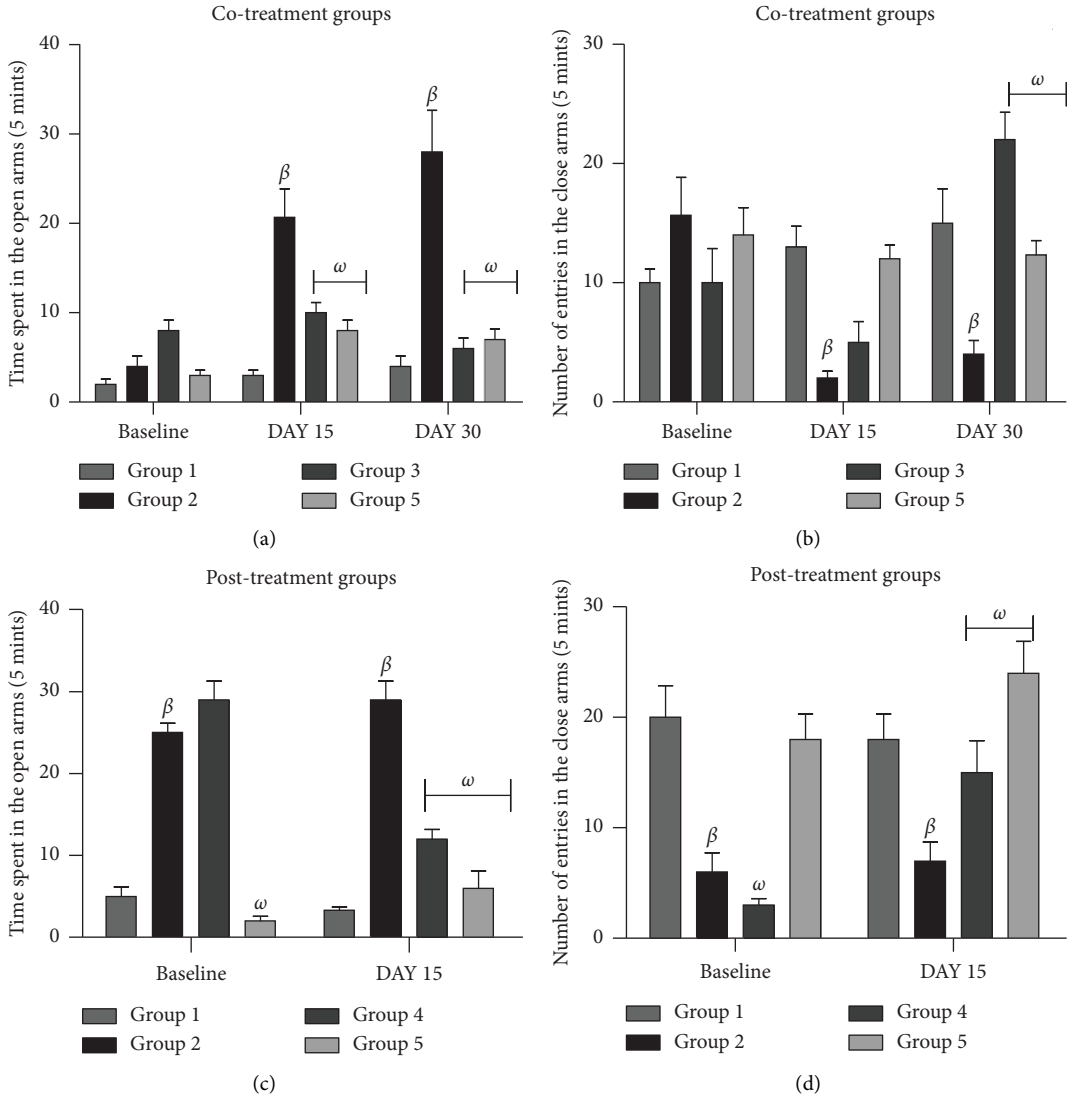


Figure 2: Effect of *S. mombin* (100 mg/kg) on anxiety-like behavioural disturbances following copper-induced brain toxicity as ascertained with elevated mazed plus paradigm. (a and c) Difference in close-arm time. (b) The (d) number of visits into the open arms followed the co and postadministration with the aqueous plant extract on days 30 and 15 respectively. Given that the statistical data were expressed as mean ± SD ($n = 6$). β represents a significant difference between the $CuSO_4$-induced group (group 2, i.e., without treatment) and the control, ω represents a significant ($p < 0.05$) difference between the treated groups (groups 3 and 4) and Cu-induced group. Group 1: control; group 2: $CuSO_4$ group only; group 3: cotreatment group; group 4: posttreatment group; group 5: treated with extract alone.

3.4. Residual Copper Concentration in the Brain and Blood of the Mice Expose to Copper Sulphate. This study clearly showed that copper ion concentration in the male mice brains increased significantly (Table 2).

3.5. Effect of S. mombin Methanolic Extract on Oxidative Stress and Enzymes Associated With ACH Metabolism

3.5.1. S. mombin Ameliorates the Activity of AChE, BuChE and Choline Acetyltransferase In Vivo. Figure 3 presents the inhibitory and ameliorative action of *S. mombin* on cholinergic enzyme activities, and in both co- and posttreatment trials, it could be deduced that copper exposure resulted in a significant ($p < 0.05$) increase in AChE activity, while, on the contrary, resulted in significant decrease ($p < 0.05$) in

Table 2: Copper ion concentration in male mice blood and brain following copper sulphate exposure.

Treatment	Blood copper concentrations (ng/g of tissue)	Brain copper concentration (ng/g of tissue)
Group 1	0.17 ± 0.008	0.05 ± 0.0023
Group 2	78 ± 3.85*	24.23 ± 0.092
Group 3	30.56 ± 11.63[a]	11.69 ± 0.012[a]
Group 4	42.14.69 ± 0.046[a]	27 ± 0.019[a]
Group 5	0.12 ± 0.99[a]	0.089.89 ± 0.003[a]

Note: group 1: (control group); group 2: ($CuSO_4$ group only); group 3: (cotreatment group); group 4: (posttreatment group); group 5: (treated with extract alone).

Data represented as mean ± SD ($n = 4$). Bars marked *are significantly different from the control group ($p < 0.05$) while those marked "a" are significantly ($p > 0.05$) different from the copper group.

BuChE and choline acetyltransferase (Figures 3(a), 3(b) and 3(c)). However, our investigation shows that treatment with the extracts of *S. mombin* at 100 mg/kg reversed this trend ($p < 0.05$), and these values were not significantly different from the control group which is an indication of normalization ($p > 0.05$). It could be safely suggested that the reduced AChE activity could have resulted in increased levels of ACH at the synapse which in turn abrogated the cognitive decline resulting from copper exposure (Scheme 2).

3.5.2. Attenuation of Oxidative Stress in the Brain of Male Mice by S. mombin Following Exposure to Copper Sulphate. Our investigations confirmed a marked increase ($p < 0.05$) in lipid peroxidation in mice exposed to copper sulphate compared to the control (4A). This was followed by a marked reduction ($p < 0.05$) in glutathione transferase and CAT activities, as well as a decrease in total thiol level (Figures 4(b), 4(c) and 4(e)). Although, the Cu-induced group showed a reduction SOD activity this is insignificant to the control group (4D). However, the decrement in antioxidant enzymes and total thiol level was overturned after co- and posttreatment with *S. mombin*. There was also a significant decrease in lipid peroxidation level indicating that the administration of *S. mombin* at 100 mg/kg resulted in a considerable reduction in oxidative stress induced by copper sulphate exposure (Scheme 2).

3.6. Histological Studies Results

3.6.1. Treatment With S. mombin Increased Neuronal Density in the Hippocampal Areas of Mice. As depicted in Figures 5(a), 5(b) and 5(c) (Figures 6, 7 and 8), respectively, Cu-induced kindling lowers the number of pyramidal neurons and vacuolated nerve cells in the hippocampal location cornu ammonis (CA1 and CA3) (Figures 6 and 7). The hippocampus neutrons in the Cu-induced mice group are substantially lesser than those of control mice. Furthermore, *S. mombin* extract increased the relative numbers of nerve cell processes in the CA1 and CA3 areas (Figures 5(a) and 5(b)) and improved Morris's water cognitive performance (Figure 1 and Scheme 2) in both post and cotreatment animals at 100 mg/kg of oral intake of the extract and eliminated the negative action of cognitive impairment in the hippocampus-dependent CA1 and CA3 area in albino male mice.

4. Discussion

Environmental copper exposures have been implicated in the aetiology of several neurological disorders and impaired cognitive abilities. The particular mechanisms of its effects on cognitive function are controversial and not well understood. We therefore unveil the importance of SML extract in mitigating the toxic insult mediated by copper on the cholinergic system via oral gavage. The elevated copper concentration observed in this study is an indication that copper could pass through the blood–brain barrier and could elicit its neurotoxicity effects. Previous studies support our findings and show that heavy metals could pass through the blood–brain barrier and accumulate in the brain [49]. Previous investigation has also related its accumulation to blood–brain barrier breakdown, which may play a crucial role in neurodegeneration conditions [49].

Our result shows learning and memory skills deficits as presented as a significant increase in escape latency (Figure 4(a)) and reference memory (Figure 4(b)) in the copper-treated group compared with control. The results of the present study were in agreement with the previous studies by Lamtai et al. [50] who reported that chronic Cu exposure could impair working memory in rat-induced models via cholinergic dysfunctions. The study also showed a correlation with the studies of Zhang et al. [51] and Zhang et al. [13], who demonstrated that high levels of Cu could accumulate in the brain tissues and could impair memory and cognitive performance via the decrease in glutamate levels, oxidative damage and cell death. Another finding of this investigation was linked with increased anxiety-like behavioural disturbance (Scheme 2), and this was depicted by an increase in the number of entries and time spent in the open arms in the EPM behavioural assessment. The findings of our study also align with the research conducted by Lamtai et al. [50], which supports the notion that the treatment with Cu could induce anxiety-like behaviours in rats. On the other hand, *S. mombin* at 100 mg/kg abrogated this effect. Previous studies support our finding and showed that *S. mombin* could modulate behavioural changes in rats' models [52, 53]. The above action of *S. mombin* could be due to the inhibitory potential of AChE crucial enzyme in ACH catabolism (Scheme 2).

The observed increase in cognition deficits in the Cu groups was correlated with an increased AChE activity. The key finding of these studies also presents a decrease in BuChE and choline acetyltransferase activities (Scheme 2). It could be that one of the primary mechanisms used by Cu to elicit its toxicity is through the alterations in cholinergic enzymes at the synaptic cleft. Previous studies have also related copper's harmful effect to impair memory performance by hindering ACH action at the synapse and partially through the alteration in cholinergic enzyme activities [54, 55]. This evidence indicates that the disturbances in learning and other aspects of behaviour following Cu exposure may be related to significant changes in CNS cholinergic enzymes, the proteins that metabolise excitatory neurotransmitters (ACH). One possible mechanism of Cu's toxicity on ACHE activity may be the action of Cu with certain amino acid residues in the active site of the enzyme via an electrostatic interaction [56]. It is widely assumed that ACH is a presynaptic modulator and plays a pivotal role in learning and memory processing [24]. Its deficiencies have been linked to higher cognitive impairment as seen in NDs [18, 20]. However, results from this study showed that the plant extracts (100 mg/kg *S. mombin*) were able to inhibit AChE activity. Furthermore, in a separate instance, the extract demonstrated a substantial augmentation in choline acetyltransferase activity. Our result was in agreement with the previous report which also demonstrated the inhibitory action of *S. mombin* on AChE activity [53, 57]. We could safely suggest that the reduced AChE activity and increased choline acetyltransferase activity could have resulted in increased levels of ACH at the synapse which in

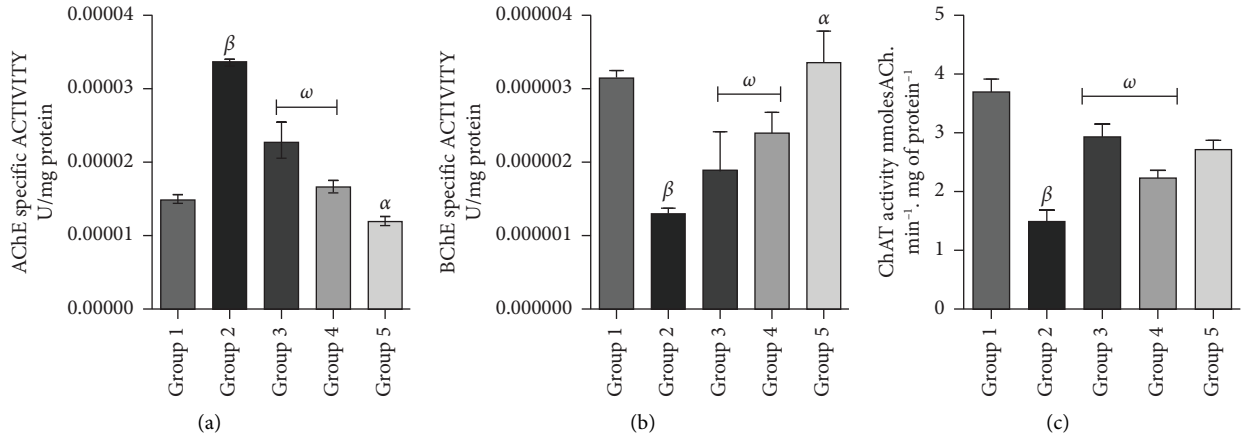


FIGURE 3: The beneficial effect of *S. mombin* administration on acetylcholinesterase (a), butyrylcholinesterase (b and c) choline acetyltransferase, activities followed $CuSO_4$-induced perturbation in mice brains. Given that the statistical data were expressed as mean ± SD ($n = 4$). β represents a significant difference between the $CuSO_4$-induced group (group 2, i.e., without treatment) and the control, and ω represents a significant ($p < 0.05$) difference between the treated groups (groups 3 and 4) and Cu-induced group, while α shows that there are no differences ($p > 0.05$) between the group treated with the extract alone and control. Group 1: control group; group 2: $CuSO_4$ group only; group 3: cotreatment group; group 4: posttreatment group; group 5: treated with extract alone.

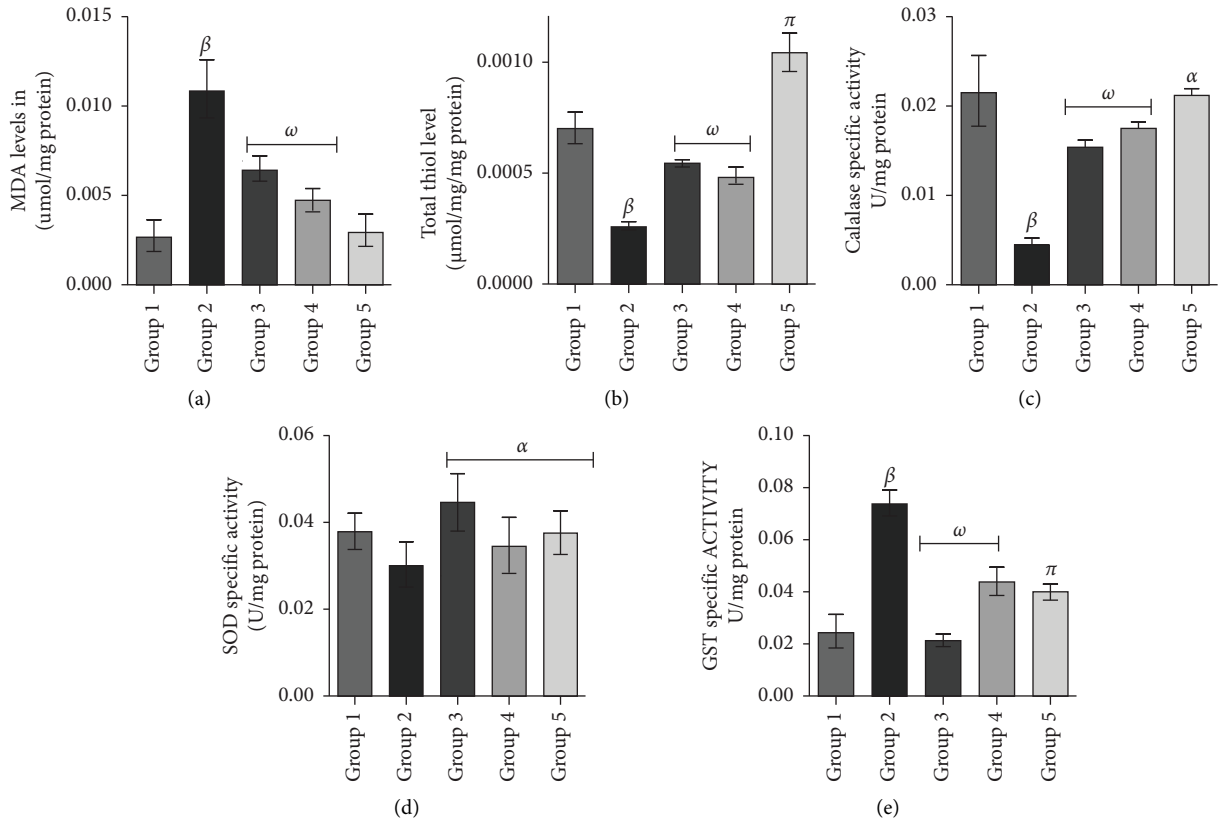


FIGURE 4: *S. mombin* lipid peroxidation (LPO) effect as a result of damage to the mice brain followed by oral copper administration. Antioxidants measured in this regard include total thiol group, glutathione transferase (GST), catalase and superoxide dismutase (SOD). LPO levels were also determined. Given that the statistical data were expressed as mean ± SD ($n = 4$). β represents a significant difference between the $CuSO_4$-induced group (group 2, i.e., without treatment) and the control, ω represents a significant ($p < 0.05$) difference between the treated groups (groups 3 and 4) and Cu-induced group and π represents a significant difference between the groups treated with the extra alone and the control group, while α shows that there are no differences ($p > 0.05$) between the group treated with the extract alone and control.

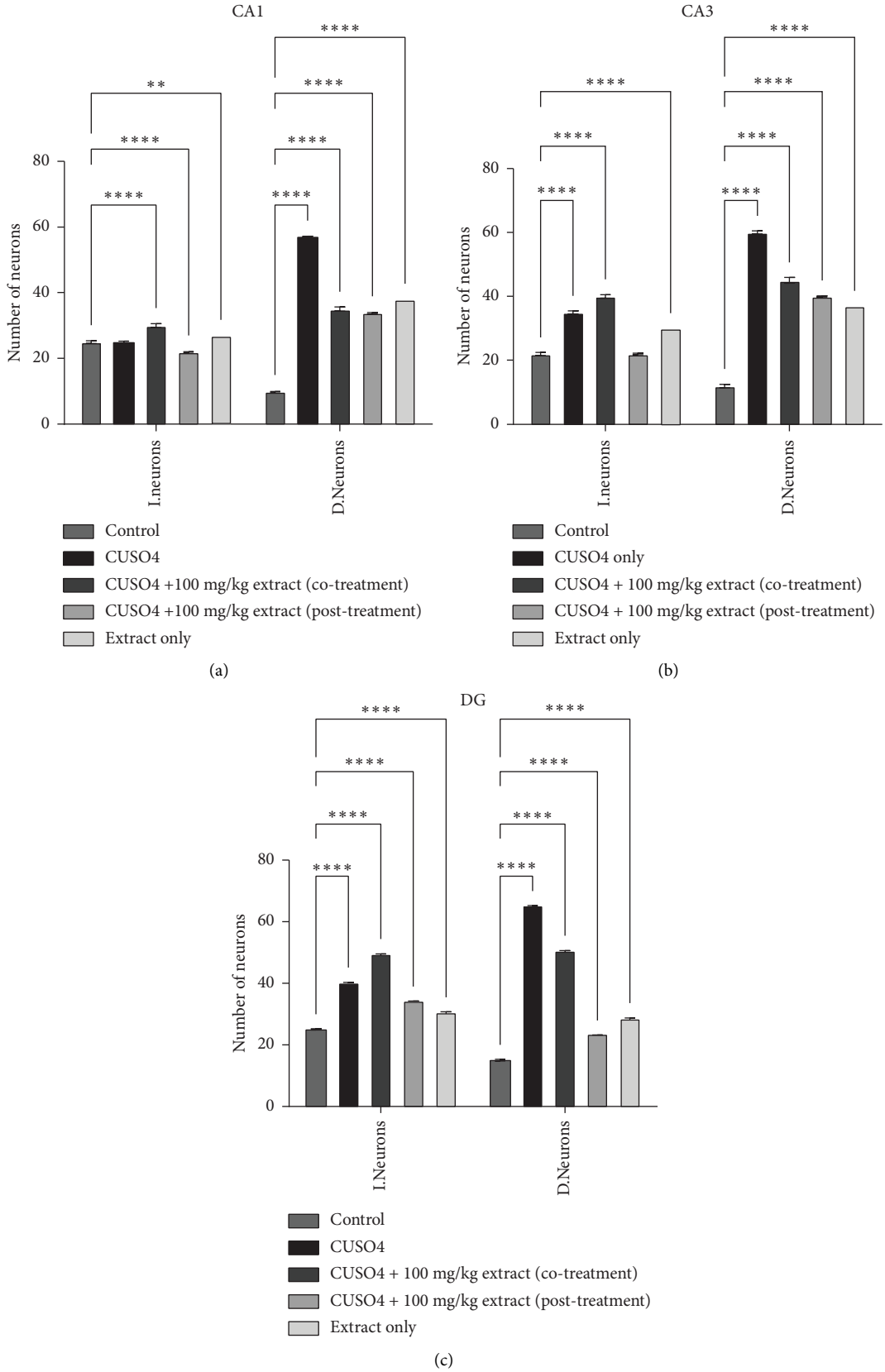


Figure 5: The benefits of *S. mombin* crude extracts on hippocampal subfields and learning deficit in Cu-induced mice were experimentally investigated. (a) Bar chart showing the neuronal count of the CA1 hippocampal subfield, (b) a bar chart showing the neuronal count of the CA3 hippocampal subfield and (c) a bar chart showing the neuronal count of the DG hippocampal subfield. $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$. Significant difference when comparison was done within the groups. Values are expressed as mean ± SEM $n = 2$, D. Neurons = neurons showing degenerating features, I. Neurons = intact neurons.

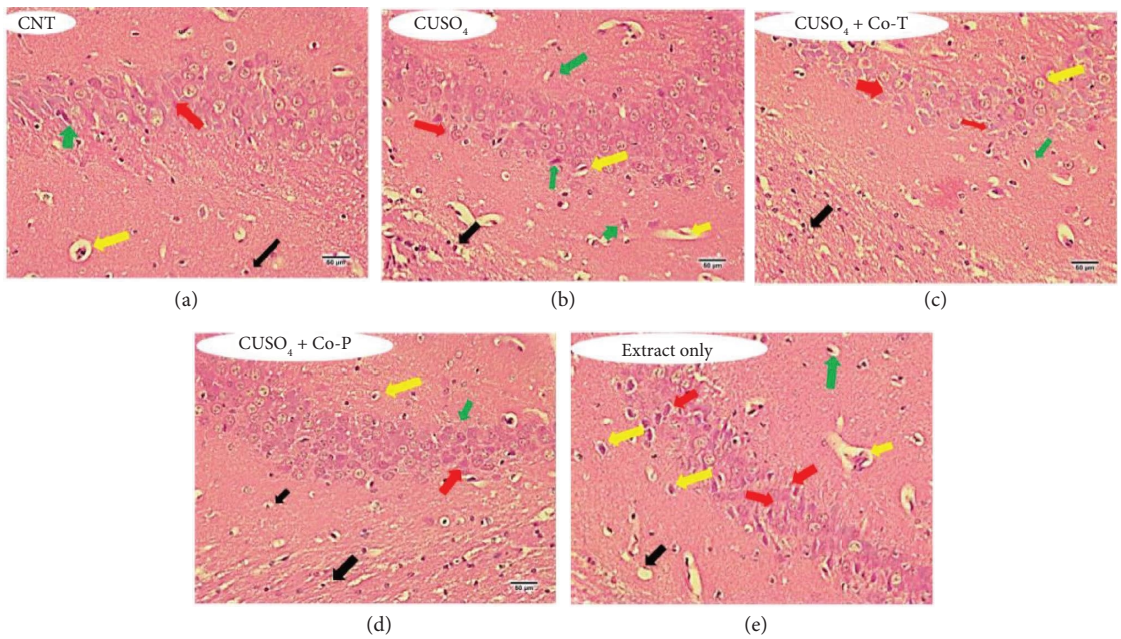


Figure 6: Representative photomicrographs of sections of hippocampal CA1 subfield subjected to H&E stain. The upper panel represents (a) control and (b) $CUSO_4$ (induced GRP without treatment). The lower panel represents (c) $CUSO_4$ + extract (cotreatment with extract) and (d) $CUSO_4$ + extract (posttreatment with the extract), while (e) where treated with the extract alone (green arrow)—degenerating (pyknotic) neuron, (yellow arrow) vacuolated neuron, (red arrow)—intact neuron (pyramidal cell), (black arrow)—oligodendrocyte.

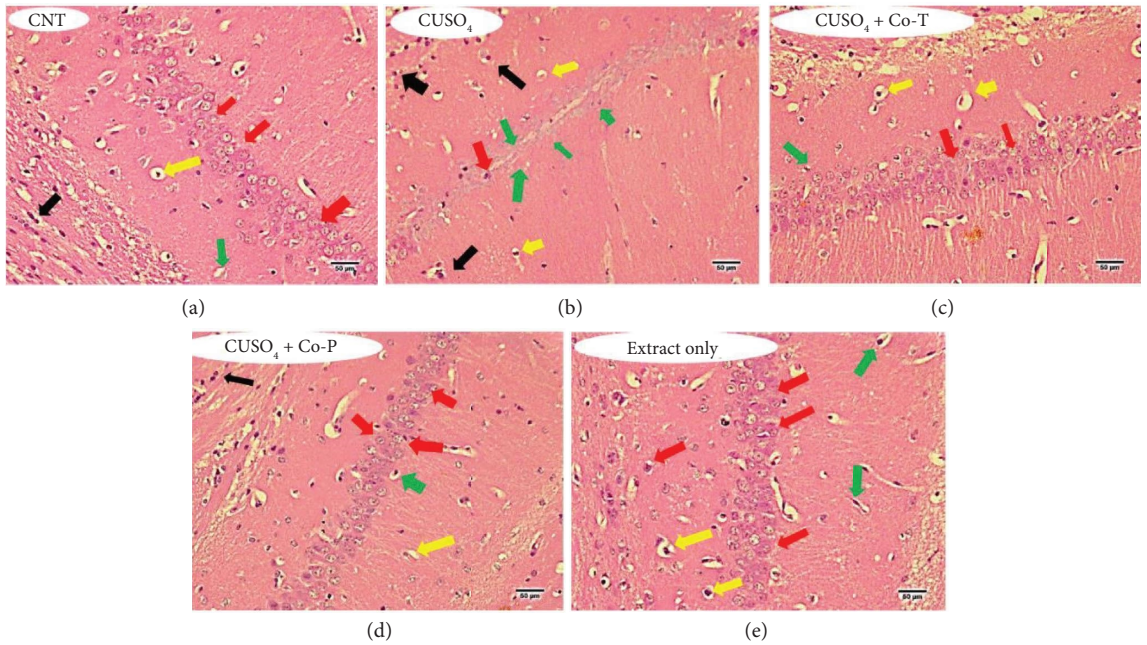


Figure 7: Representative photomicrographs of sections of hippocampal CA3 subfield subjected to H&E stain. The upper panel represents (a) control and (b) $CUSO_4$ (induced GRP without treatment). The lower panel represents (c) $CUSO_4$ + extract (cotreatment with extract) and (d) $CUSO_4$ + extract (posttreatment with the extract), while (e) where treated with the extract alone (green arrow)—degenerating (pyknotic) neuron, (yellow arrow) vacuolated neuron, (red arrow)—intact neuron (pyramidal cell), (black arrow)—oligodendrocyte.

turn abrogated the cognitive decline resulting from Cu exposure. The antioxidant property and alkaloids present in the extract may be contributing favourably to the AChE inhibition activity observed [57].

The integrity of the central nervous system may be compromised by oxidative stress, which in turn can have an impact on cognitive performance [58]. Our study demonstrated an increase in lipid peroxidation levels following Cu-induced neurotoxicity. The elevated LPO levels caused by Cu in hippocampal tissue are accompanied by a significant reduction in GST, SOD, and CAT activities (Scheme 2). This is an indication of oxidative stress. These findings align with the studies

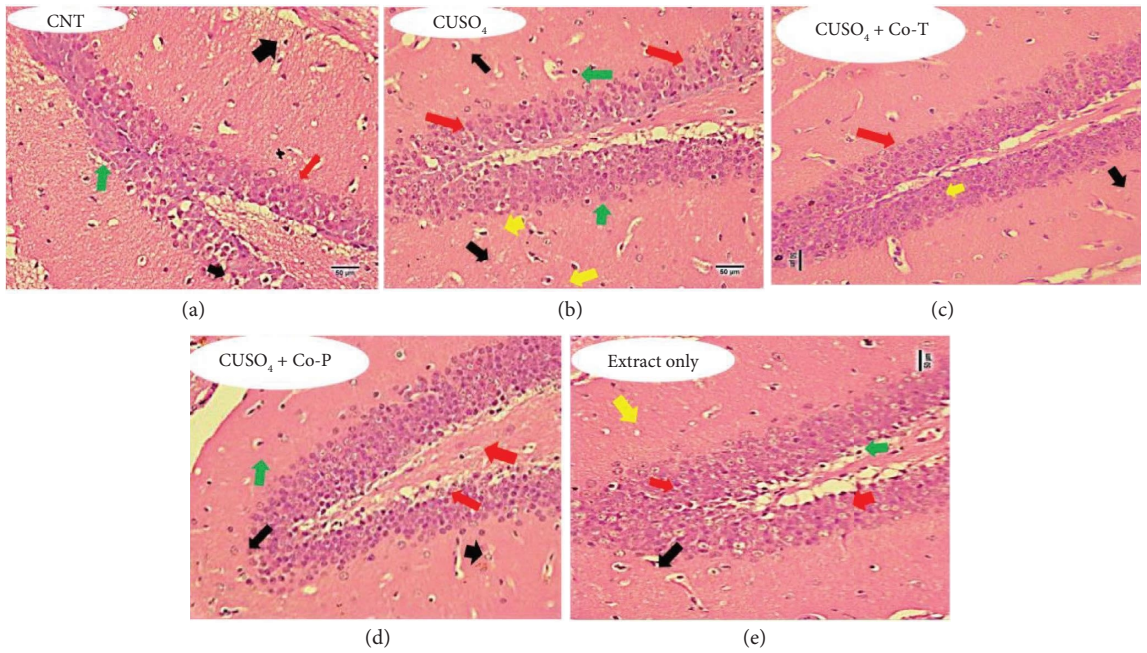


Figure 8: Representative photomicrographs of sections of hippocampal DG subfield subjected to H&E stain. The upper panel represents (a) control and (b) $CUSO_4$ (induced GRP without treatment). The lower panel represents (c) $CUSO_4$ + extract (cotreatment with extract) and (d) $CUSO_4$ + extract (posttreatment with the extract), while (e) where treated with the extract alone (green arrow)—degenerating (pyknotic) neuron, (yellow arrow) vacuolated neuron, (red arrow)—intact neuron (pyramidal cell), (black arrow)—oligodendrocyte.

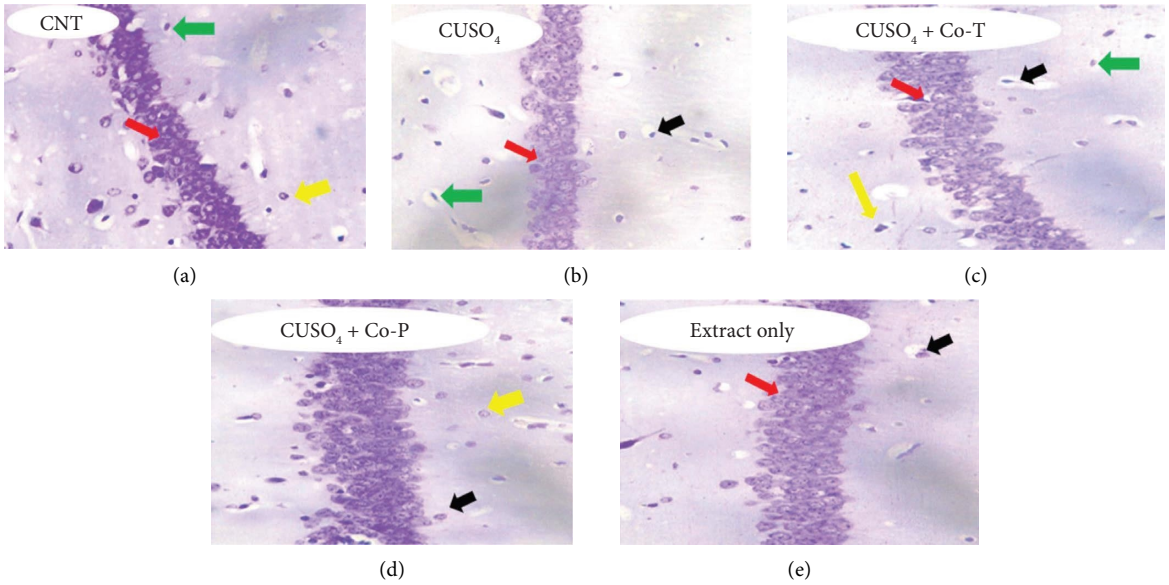


Figure 9: Representative photomicrographs of sections of hippocampal CA1 sub-field subjected to cresyl fast violet stain. The upper panel represents (a) control and (b) $CUSO_4$ (induced GRP without treatment). The lower panel represents (c) $CUSO_4$ + extract (cotreatment with extract) and (d) $CUSO_4$ + extract (posttreatment with the extract), while (e) where treated with the extract alone (green arrow)—degenerating (pyknotic) neuron, (yellow arrow) vacuolated neuron, (red arrow)—intact neuron (pyramidal cell), (black arrow)—oligodendrocyte.

conducted by Behzadfar et al. and Kalita et al., which showed that Cu treatment increased damage and caused changes in the enzymatic antioxidant defence state [59, 60]. Also, this study reported a significant decrease in total thiol levels. The reasoning for decreased total thiol levels observed in this study could be that Cu binds to total thiol sulfhydryl groups and renders them inactive to neutralized ROS. Surprisingly, treatment with 100 mg/kg extracts of *S. mombin* significantly increased the antioxidant enzyme activities and as well lowered lipid peroxidation and elevated total thiol levels (Scheme 2). These findings align with some other studies that attest that medicinal plants are abundant in exogenous antioxidant

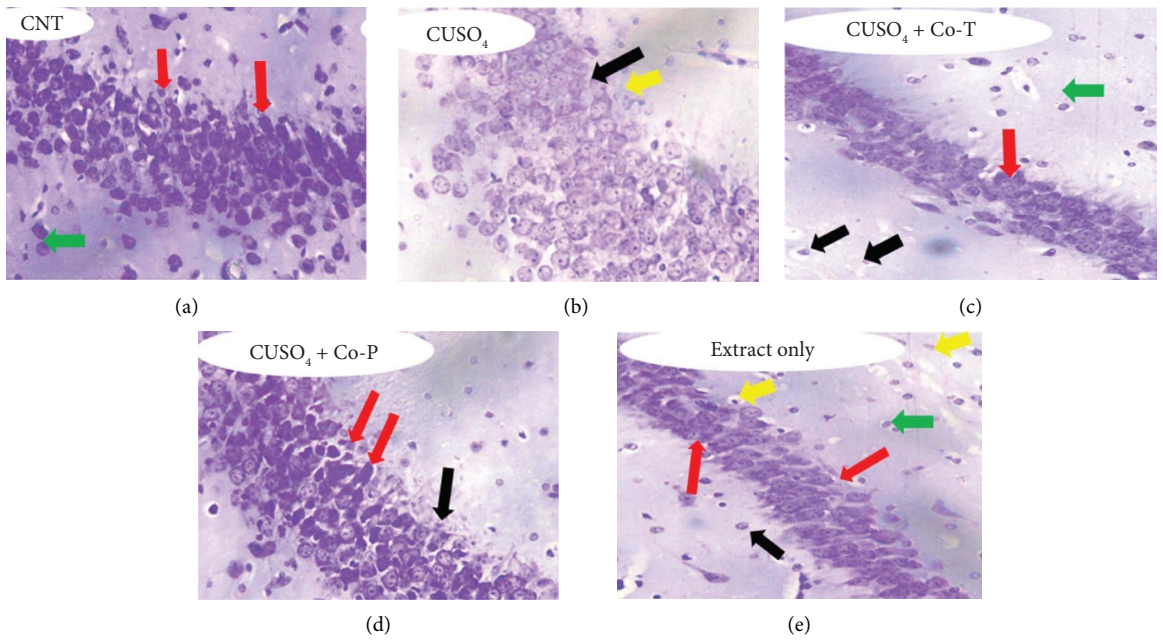


Figure 10: Representative photomicrographs of Sections of hippocampal CA3 sub-field subjected to cresyl fast violet stain. The upper panel represents (a) control and (b) $CUSO_4$ (induced GRP without treatment). The lower panel represents (c) $CUSO_4$ + extract (cotreatment with extract) and (d) $CUSO_4$ + extract (posttreatment with the extract), while (e) where treated with the extract alone (green arrow)—degenerating (pyknotic) neuron, (yellow arrow) vacuolated neuron, (red arrow)—intact neuron (pyramidal cell), (black arrow)—oligodendrocyte.

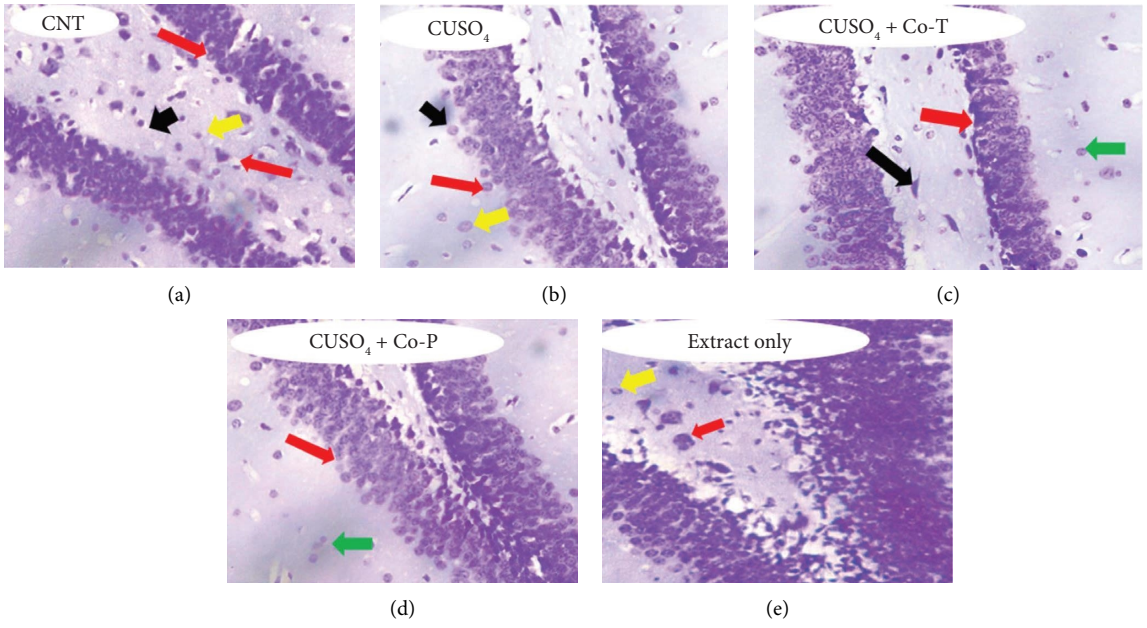


Figure 11: Representative photomicrographs of Sections of hippocampal DG sub-field subjected to cresyl fast violet stain. The upper panel represents (a) control and (b) $CUSO_4$ (induced GRP without treatment). The lower panel represents (c) $CUSO_4$ + extract (cotreatment with extract) and (d) $CUSO_4$ + extract (posttreatment with the extract), while (e) where treated with the extract alone (green arrow)—degenerating (pyknotic) neuron, (yellow arrow) vacuolated neuron, (red arrow)—intact neuron (pyramidal cell), (black arrow)—oligodendrocyte.

defences such as polyphenols and have the potential to safeguard cognitive function through the modulation of oxidative stress markers (Scheme 2), therefore enhancing spatial learning and memory [5, 61]. It has been demonstrated in this study that *S. mombin* improves cognitive impairment by altering the levels of oxidative stress markers. The antioxidant properties present in these plants may be contributing favourably to the increase of these activities.

In addition to biochemical alterations, the hippocampus regions (CA1, CA3 and DG), which are mostly involved in memory formation [62], showed a notable loss of neurons after $CUSO_4$-induced toxicity, and these regions are primarily involved in memory formation. Therefore, behavioural deficits observed in this study are linked to these alterations (Scheme 2). On the other hand, treatment with *S. mombin* reduced such changes while maintaining the normal neuron count. This was consistent with the findings of Elufioye and Oyelude [63], who discovered that *S. mombin* plant extract could increase neuronal cell density in the CAI and CA3 regions of the hippocampus [63]. Furthermore, the cresyl violet staining shows several features of pyramidal neuronal degeneration with decreased Nissl substance in the copper-induced group (Figures 9, 10 and 11). In contrast, treatment with *S. mombin* showed normal neuronal cell bodies with intact Nissl substance which indicated improved degenerate pyramidal neurons after copper toxicity exposure. Nissl substances are an integral component of the rough endoplasmic reticulum where a protein synthesis takes place [64], so, therefore, an increase in Nissl substance activity observed could indicate increased production of intercellular protein molecules that function to improve neuronal cell structure.

5. Conclusion

S. mombin ameliorates Cu-induced neurotoxicity and neurocognitive dysfunctions by virtue of enhancement of cholinergic signalling in the brain. Moreover, *S. mombin* exhibits antioxidant activities by up-regulating antioxidant levels in the brain. Therefore, *S. mombin* may have the potential to be used as a therapeutic intervention for the management of Cu-induced cognitive impairment.

Nomenclature

AChE	Acetylcholinesterase
AD	Alzheimer's disease
BSA	Bovine serum albumin
BuChE	Butyrylcholinesterase
CA1 and CA3	Hippocampal location cornu ammonis
CDNB	1-Chloro-2, 4-dinitrobenzene
Cu	Copper
DNTB	5,5″-Dithiol-bis (2-nitrobenzoic) acid
EPM	Elevated plus-maze
MWM	Morri's water maze task
NFTs	Neurofibrillary tangles
EDTA	Ethylenediaminetetraacetic acid
S. mombin	*Spondias mombin*
ND	Neurodegenerative disorders
ROS	Reactive oxygen species
ACH	Acetylcholine

Data Availability Statement

The data generated during the study will be provided upon a reasonable request from the corresponding author.

Ethics Statement

The animals used were by the rules and guidance of Obafemi Awolowo University's College of Health Sciences in Ile-Ife, Osun State, Nigeria.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

The authors confirm sole responsibility for the following:

J.B.A.: conceived the presented idea, study conception and design, experimented, writing—original draft preparation, analysis and interpretation of results, resources, specifically visualization/data presentation and provision of study materials.

A.R.J.: study conception and design, conducting research and investigation process, provision of study materials and reviewing and editing.

S.T.O.: data curation and conceptualization, software and reviewing and editing.

J.O.O.: reviewing and editing.

A.F.E.: supervision and resources.

O.O.O.: supervision and resources.

All authors provided critical feedback and helped shape the research, analysis and manuscript. Also, all authors read and approved the final manuscript.

Funding

There was no particular grant for this research from any funding body in the public, private, or any other domain.

Acknowledgements

The authors would like to express their gratitude to Mr Ilesanmi of the Department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife, Nigeria, for his scientific advice and assistance. We acknowledge the help of Grammarly in improving the writing and nothing but the writing of this article.

References

[1] P. Georgopoulos, S. Wang, I. Georgopoulos, M. Yonone-Lioy, and P. Lioy, "Assessment of Human Exposure to Copper: A Case Study Using the NHEXAS Database," *Journal of Exposure Science and Environmental Epidemiology* 16, no. 5 (2016): 397–409.

[2] B. R. Stern, "Essentiality and Toxicity in Copper Health Risk Assessment: Overview, Update and Regulatory Considerations," *Journal of Toxicology and Environmental Health, Part A* 73, no. 2-3 (2010): 114–127, https://doi.org/10.1080/15287390903337100.

[3] M. Bost, S. Houdart, M. Oberli, E. Kalonji, J. F. Huneau, and I. Margaritis, "Dietary Copper and Human Health: Current Evidence and Unresolved Issues," *Journal of Trace Elements in Medicine & Biology* 35 (2016): 107–115, https://doi.org/10.1016/j.jtemb.2016.02.006.

[4] S. A. M A Noor, N. Salman, and K. H. Al-Naimi, "Determination the Concentrations of Heavy Metals in the Blood of Cement Plant Workers in Kufa and Compared with the Other Workers: An Impact on Biological Processes in the Human Body," *Annals of Tropical Medicine and Public Health* 24, no. 04 (2021): 382–393, https://doi.org/10.36295/asro.2021.24443.

[5] K. C. Spitalny, J. Brondum, R. L. Vogt, H. E. Sargent, and S. Kappel, "Drinking-Water-Induced Copper Intoxication in a Vermont Family," *Pediatrics* 74, no. 6 (1984): 1103–1106, https://doi.org/10.1542/peds.74.6.1103.

[6] S. J. More, V. Bampidis, D. Benford, et al., "Re-evaluation of the Existing Health-based Guidance Values for Copper and Exposure Assessment from All Sources," *EFSA Journal* 21, no. 1 (2023): 07728, https://doi.org/10.2903/j.efsa.2023.7728.

[7] E. Commisssion, "Opinion of the Scientific Committee on Food on the Tolerable Upper Intake Level of Copper (SCF/CS/NUT/GEN/18), Brussels," (2003), https://food.ec.europa.eu/system/files/2020-12/sci-com_scf_out171_en.pdf.

[8] H. W. Hsu, S. C. Bondy, and M. Kitazawa, "Environmental and Dietary Exposure to Copper and Its Cellular Mechanisms Linking to Alzheimer's Disease," *Toxicological Sciences* 163, no. 2 (2018): 338–345, https://doi.org/10.1093/toxsci/kfy025.

[9] H. Ejaz, W. Wang, and M. Lang, "Copper Toxicity Links to Pathogenesis of Alzheimer's Disease and Therapeutics Approaches," *International Journal of Molecular Sciences* 21, no. 20 (2020): 7660, https://doi.org/10.3390/ijms21207660.

[10] P. S. Donnelly, Z. Xiao, and A. G. Wedd, "Copper and Alzheimer's Disease," *Current Opinion in Chemical Biology* 11, no. 2 (2007): 128–133, https://doi.org/10.1016/j.cbpa.2007.01.678.

[11] G. Gromadzka, B. Tarnacka, A. Flaga, and A. Adamczyk, "Copper Dyshomeostasis in Neurodegenerative Diseases—Therapeutic Implications," *International Journal of Molecular Sciences* 21, no. 23 (2020): 9259, https://doi.org/10.3390/ijms21239259.

[12] P. Georgopoulos, S. Wang, I. Georgopoulos, M. Yonone-Lioy, and P. Lioy, "Assessment of Human Exposure to Copper: A Case Study Using the NHEXAS Database," *Journal of Exposure Science and Environmental Epidemiology* 16, no. 5 (2006): 397–409, https://doi.org/10.1038/sj.jes.7500462.

[13] Y. Zhang, Q. Zhou, L. Lu, et al., "Copper Induces Cognitive Impairment in Mice via Modulation of Cuproptosis and CREB Signaling," *Nutrients* 15, no. 4 (2023): 972, https://doi.org/10.3390/nu15040972.

[14] K. Jomova, M. Cvik, P. Lauro, et al., "The Role of Redox Active Copper (II) on Antioxidant Properties of the Flavonoid Baicalein: DNA Protection under Cu (II)-Fenton Reaction and Cu (II)-ascorbate System Conditions," *Journal of Inorganic Biochemistry* 245 (2023): 112244, https://doi.org/10.1016/j.jinorgbio.2023.112244.

[15] X. Li, S. Hao, A. Han, et al., "Intracellular Fenton Reaction Based on Mitochondria-Targeted Copper (ii)–peptide Complex for Induced Apoptosis," *Journal of Materials Chemistry B* 7, no. 25 (2019): 4008–4016, https://doi.org/10.1039/c9tb00569b.

[16] T. Pereira, M. Campos, and M. Bogo, "Copper Toxicology, Oxidative Stress and Inflammation Using Zebrafish as Experimental Model," *Journal of Applied Toxicology* 36, no. 7 (2016): 876–885, https://doi.org/10.1002/jat.3303.

[17] M. Kitazawa, H. Hsu, and R. Medeiros, "Copper Exposure Perturbs Brain Inflammatory Responses and Impairs Clearance of Amyloid-Beta," *Toxicological Sciences* 152, no. 1 (2016): 194–204, https://doi.org/10.1093/toxsci/kfw081.

[18] N. Greig, T. Utsuki, Q. Yu, et al., "A New Therapeutic Target in Alz-Heimer's Disease Treatment: Attention to Butyrylcholinesterase," *Current Medical Research and Opinion* 17, no. 3 (2001): 159–165, https://doi.org/10.1185/0300799039117057.

[19] R. Cools and A. Arnsten, "Neuromodulation of Prefrontal Cortex Cognitive Function in Primates: The Powerful Roles of Monoamines and Acetylcholine," *Neuropsychopharmacology* 47, no. 1 (2022): 309–328, https://doi.org/10.1038/s41386-021-01100-8.

[20] Y. Ha, S. Mathew, and K. Yeong, "Butyrylcholinesterase: A Multifaceted Pharmacological Target and Tool," *Current Protein & Peptide Science* 21, no. 1 (2020): 99–109, https://doi.org/10.2174/1389203720666191107094949.

[21] A. E. Al-Snafi, "Medicinal Plants with Cholinesterase Inhibitory Activity and Their Applications," *GSC Biological and Pharmaceutical Sciences* 19, no. 1 (2022): 235–248, https://doi.org/10.30574/gscbps.2022.19.1.0154.

[22] H. Hampel, M. Mesulam, A. C. Cuello, et al., "The Cholinergic System in the Pathophysiology and Treatment of Alzheimer's Disease," *Brain* 141, no. 7 (2018): 1917–1933, https://doi.org/10.1093/brain/awy132.

[23] A. Baradaran, Z. Rabiei, M. Rafieian, and H. Shirzad, "A Review Study on Medicinal Plants Affecting Amnesia through Cholinergic System," *Journal of HerbMed Pharmacology* 1, no. 1 (2012): 3–9.

[24] S. Bhadra, M. K. Dalai, J. Chanda, and P. K. Mukherjee, "Evaluation of Bioactive Compounds as Acetylcholinesterase Inhibitors From Medicinal Plants," *Evidence-Based Validation of Herbal Medicine* 11 (2015): 273–306, https://doi.org/10.1016/b978-0-12-800874-4.00013-1.

[25] A. Mondal, R. Bhar, and S. N. Sinha, "Ethnomedicinal Value and Biological Activities of *Spondias mombin* L-A Concise Review," *Asian Research Journal of Current Science* 13, no. 1 (2021): 87–94.

[26] O. Abiodun, M. E. Nnoruka, and R. Tijani, "Phytochemical Constituents, Antioxidant Activity, and Toxicity Assessment of the Seed of *Spondias mombin L.* (Anacardiaceae)," *Turkish Journal Of Pharmaceutical Sciences* 17, no. 3 (2020): 343–348, https://doi.org/10.4274/tjps.galenos.2020.38801.

[27] D. Kumadoh, M. Archer, G. Yeboah, et al., "A Review on Anti-Peptic Ulcer Activities of Medicinal Plants Used in the Formulation of Enterica, Dyspepsia and NPK 500 Capsules," *Heliyon* 7, no. 12 (2021): e08465–65, https://doi.org/10.1016/j.heliyon.2021.e08465.

[28] S. Saha, "Phytochemical Screening and Comparative Study of Antioxidative Properties of the Fruits and Leaves of Spondias Mombin in Bangladesh," *Journal of Pharmacognosy and Phytochemistry* 8, no. 2 (2019): 379–383.

[29] E. Hussein, A. Taj-Eldeen, A. Al-Zubairi, A. S. Elhakimi, and A. Al-Dubaie, "Phytochemical Screening, Total Phenolics and Antioxidant and Antibacterial Activities of Callus From Brassica Nigra L. Hypocotyl Explants," *International Journal of Pharmacology* 6, no. 4 (2010): 464–471, https://doi.org/10.3923/ijp.2010.464.471.

[30] P. Tiwari, B. Kumar, M. Kaur, G. Kaur, and H. Kaur, "Phytochemical Screening and Extraction, A Review," *International Journal of Pharmacy and Pharmaceutical Sciences* 1, no. 1 (2011): 98–106.

[31] O. O. Oyedapo, A. M. Makinde, G. M. Ilesanmi, E. O. Abimbola, K. Akinwunmi, and B. Akinpelu, "Biological Activities (Anti-Inflammatory and Anti-Oxidant) of Fractions and Methanolic Extract of *Philonotis hastate* (Duby Wijk & MargaDant)," *African Journal of Traditional,*

Complementary and Alternative Medicines 12, no. 4 (2015): 50–55, https://doi.org/10.4314/ajtcam.v12i4.8.

[32] A. Meda, C. Lamien, M. Romito, J. Millogo, and O. Nacoulma, "Determination of the Total Phenolic, Flavonoid and Proline Contents in Burkina Fasan Honey, as Well as Their Radical Scavenging Activity," *Food Chemistry* 91, no. 3 (2005): 571–577, https://doi.org/10.1016/j.foodchem.2004.10.006.

[33] H. Liu, H. Guo, Z. Jian, et al., "Copper Induces Oxidative Stress and Apoptosis in the Mouse Liver," *Oxidative Medicine and Cellular Longevity* 2020 (2020): 1–20, https://doi.org/10.1155/2020/1359164.

[34] J. Kumar, K. B. Sathua, and S. J. S. Flora, "Chronic Copper Exposure Elicit Neurotoxic Responses in Rat Brain: Assessment of 8-Hydroxy-2-Deoxyguanosine Activity, Oxidative Stress and Neurobehavioral Parameters," *Cellular and Molecular Biology* 65, no. 1 (2019): 27–35, https://doi.org/10.14715/cmb/2019.65.1.5.

[35] O. E. Ola-Davies, T. O. Ajibade, E. Samuel, and R. A. Adedokun, "Cytoprotective Potential of Spondias Mombin Fractions on Sodium Arsenite-Induced Haematological, Biochemical and Genotoxic Perturbations in Rats," *African Journal of Biomedical Research* 22, no. 3 (2019): 315–323.

[36] S. A. Brito, C. L. F. de Almeida, T. I. de Santana, et al., "Antiulcer Activity and Potential Mechanism of Action of the Leaves of *Spondias mombin* L," *Oxidative Medicine and Cellular Longevity* 2018, no. 1 (2018): 1731459, https://doi.org/10.1155/2018/1731459.

[37] N. Arias, C. Fidalgo, M. Méndez, and J. L. Arias, "How Demanding is the Brain on a Reversal Task Under Day and Night Conditions?" *Neuroscience Letters* 600 (2015): 153–157, https://doi.org/10.1016/j.neulet.2015.06.014.

[38] A. K. Kraeuter, P. C. Guest, and Z. Sarnyai, "The Elevated Plus Maze Test for Measuring Anxiety-Like Behavior in Rodents," *Methods in Molecular Biology* 1916 (2019): 69–74, https://doi.org/10.1007/978-1-4939-8994-2_4.

[39] C. V. Vorhees and M. Williams, "Morris Water Maze: Procedures for Assessing Spatial and Related Forms of Learning and Memory," *Nature Protocols* 1, no. 2 (2006): 848–858, https://doi.org/10.1038/nprot.2006.116.

[40] H. Garman, "Histology of the Central Nervous System," *Toxicologic Pathology* 39, no. 1 (2011): 22–35, https://doi.org/10.1177/0192623310389621.

[41] M. Bradford, "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding," *Analytical Biochemistry* 72, no. 1-2 (1976): 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.

[42] G. Ellman, K. Courtney, V. Andres, and R. Featherstone, "A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity," *Biochemical Pharmacology* 7, no. 2 (1961): 88–95, https://doi.org/10.1016/0006-2952(61)90145-9.

[43] N. Nunes-Tavares, N. L. Cunha-E-Silva, and A. Hassón-Voloch, "Choline Acetyltransferase Detection in Normal and Denervated Electrocyte from Electrophorus Electricus (L.) Using a Confocal Scanning Optical Microscopy Analysis," *Anais da Academia Brasileira de Ciências* 72, no. 3 (2000): 331–340, https://doi.org/10.1590/s0001-37652000000300007.

[44] H. Ohkawa, N. Ohishi, and K. Yagi, "Assay for Lipid Peroxides in Animal Tissues by Thiobarbituric Acid Reaction," *Analytical Biochemistry* 95, no. 2 (1979): 351–358, https://doi.org/10.1016/0003-2697(79)90738-3.

[45] G. Ellman, "Tissue Sulfhydryl Groups," *Archives of Biochemistry and Biophysics* 82, no. 1 (1959): 70–77, https://doi.org/10.1016/0003-9861(59)90090-6.

[46] W. Habig and W. Jakoby, "Assays for Differentiation of Glutathione Transferases," *Methods in Enzymology* 77 (1981): 398–405.

[47] H. Aebi, "Catalase In Vitro," *Methods in Enzymology* 105 (1984): 121–126.

[48] M. McCord and I. Fridovich, "Superoxide Dismutase," *Journal of Biological Chemistry* 244, no. 22 (1969): 6049–6055, https://doi.org/10.1016/s0021-9258(18)63504-5.

[49] N. J. Romero, M. W. Abbott, and M. W. Bradbury, "The Blood-Brain Barrier in Normal CNS and in Metal-Induced Neurotoxicity," in *Toxicology of Metals* (Boca Raton, FL: CRC Press, 2023), 561–585.

[50] M. Lamtai, O. Zghari, S. Azirar, et al., "Melatonin Modulates Copper-Induced Anxiety-Like, Depression-Like and Memory Impairments by Acting on Hippocampal Oxidative Stress in Rat," *Drug and Chemical Toxicology* 45, no. 4 (2022): 1707–1715, https://doi.org/10.1080/01480545.2020.1858853.

[51] G. Zhang, Q. Li, W. Gao, et al., "Copper Chloride Dose-Dependently Alters Spatial Learning and Memory, and Glutamate Levels, in the hippocampus of Rats," *Molecular Medicine Reports* 17 (2017): 4074–4082, https://doi.org/10.3892/mmr.2017.8278.

[52] A. M. Ajayi, B. Ben-Azu, J. C. Godson, and S. Umukoro, "Effect of Spondias Mombin Fruit Extract on Scopolamine-Induced Memory Impairment and Oxidative Stress in Mice Brain," *Journal of Herbs, Spices, & Medicinal Plants* 27, no. 1 (2021): 24–36, https://doi.org/10.1080/10496475.2020.1777613.

[53] A. O. Ademosun, T. V. Popoola, G. Oboh, and O. W. Fasakin, "*Parquetina nigrescens* and *Spondias mombin* Protects against Neurochemical Alterations in the Scopolamine Model of Cognitive Dysfunction," *Journal of Food Biochemistry* 46, no. 11 (2022): 14213, https://doi.org/10.1111/jfbc.14213.

[54] A. Pal, R. K. Badyal, R. K. Vasishta, S. V. Attri, B. R. Thapa, and R. Prasad, "Biochemical, Histological, and Memory Impairment Effects of Chronic Copper Toxicity: A Model for Non-Wilsonian Brain Copper Toxicosis in Wistar Rat," *Biological Trace Element Research* 153, no. 1-3 (2013): 257–268, https://doi.org/10.1007/s12011-013-9665-0.

[55] M. M. Elseweidy, M. Mahrous, S. I. Ali, M. A. Shaheen, and N. N. Younis, "Vitamin D Alleviates Cognitive Dysfunction and Brain Damage Induced by Copper Sulfate Intake in Experimental Rats: Focus on its Combination with Donepezil," *Naunyn-Schmiedeberg's Archives of Pharmacology* 396, no. 9 (2023): 1931–1942, https://doi.org/10.1007/s00210-023-02449-x.

[56] H. Dvir, I. Silman, M. Harel, T. L. Rosenberry, and J. L. Sussman, "Acetylcholinesterase: From 3D Structure to Function," *Chemico-Biological Interactions* 187, no. 1-3 (2010): 10–22, https://doi.org/10.1016/j.cbi.2010.01.042.

[57] T. O. Elufioye, E. M. Obuotor, J. M. Agbedahunsi, and S. A. Adesanya, "Anticholinesterase Constituents From the Leaves of *Spondias mombin* L. (Anacardiaceae)," *Biologics: Targets & Therapy* 11 (2017): 107–114, https://doi.org/10.2147/btt.s136011.

[58] J. B. Agboola, A. F. Ehigie, L. O. Ehigie, F. D. Ojeniyi, and A. A. Olayemi, "Ameliorative Role of Syzygium Aromaticum Aqueous Extract on Synaptosomal Tyrosine Hydroxylase Activity, Oxidative Stress Parameters, and Behavioral Changes in Lead-induced Neurotoxicity in Mice," *Journal of Food Biochemistry* 46, no. 7 (2022): e14115, https://doi.org/10.1111/jfbc.14115.

[59] L. Behzadfar, M. Abdollahi, O. Sabzevari, et al., "Potentiating Role of Copper on Spatial Memory Deficit Induced by Beta Amyloid and Evaluation of Mitochondrial Function Markers

in the hippocampus of Rats," *Metallomics* 9, no. 7 (2017): 969–980, https://doi.org/10.1039/c7mt00075h.

[60] J. Kalita, V. Kumar, U. K. Misra, and H. K. Bora, "Memory and Learning Dysfunction Following Copper Toxicity: Biochemical and Immunohistochemical Basis," *Molecular Neurobiology* 55, no. 5 (2018): 3800–3811, https://doi.org/10.1007/s12035-017-0619-y.

[61] B. Cabral, E. Siqueira, M. Bitencourt, et al., "Phytochemical Study and Anti-inflammatory and Antioxidant Potential of *Spondias mombin Leaves*," *Revista Brasileira de Farmacognosia* 26, no. 3 (2016): 304–311, https://doi.org/10.1016/j.bjp.2016.02.002.

[62] J. Nairismagi, A. Pitkanen, M. i. Kettunen, R. A. Kauppinen, and H. Kubova, "Status Epilepticus in 12-Day-Old Rats Leads to Temporal Lobe Neurodegeneration and Volume Reduction: A Histologic and MRI Study," *Epilepsia* 47, no. 3 (2006): 479–488, https://doi.org/10.1111/j.1528-1167.2006.00455.x.

[63] T. O. Elufioye and F. O. Oyelude, "Memory Enhancing Activity of *Spondias Mombin* Anarcadiaceae and Pycanthus Angolensis (Myristicaceae) on Scopolamine Induced Amnesia in Mice," *Nigerian Journal of Pharmaceutical Research* 11, no. 1 (2015): 84–88.

[64] M. Bhati, S. Thakre, and A. Anjankar, "Nissl Granules, Axonal Regeneration, and Regenerative Therapeutics: A Comprehensive Review," *Cureus* 15, no. 10 (2023): e47872, https://doi.org/10.7759/cureus.47872.