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Post-treatment with *Cnidoscolus aconitifolius* leaf extract ameliorates neurological and behavioral deficits following cerebral ischemia-reperfusion in rats by modulating oxidative, inflammatory, apoptotic, and neurotransmitter pathways

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Keywords: Bilateral common carotid artery occlusion, ischemic stroke, neurological deficit, neuroprotection, rat

Abstract: Oxidative and inflammatory pathways underlie the neuropathology of ischemic stroke, a neurological disease responsible for death and disability in many people worldwide. Studies showed *Cnidoscolus aconitifolius* exhibited antioxidant, anti-inflammatory, and memory-enhancing activities in rodents. However, its neuro-protective activities in post-stroke events remain poorly understood. This study sought to investigate the neuroprotective activities of an aqueous leaf extract of *Cnidoscolus aconitifolius* in rats after ischemia/reperfusion. Forty rats were assigned to five experimental groups as follows: Groups 1 (sham control) and 2 (ischemia/ reperfusion control) received distilled water (10 mL/kg), respectively. Group 3-5 had *Cnidoscolus aconitifolius* (50,100, and 200 mg/kg, p.o.) for seven days, following induction of ischemic stroke using bilateral common carotid artery occlusion (BCCAO) procedure. We evaluated neurological, motor, and memory skills on day seven following reperfusion. Oxidative stress/antioxidant biomarkers, myeloperoxidase, pro-inflammatory cytokines (TNF-α, IL-6), nuclear factor kappa B (NF-κB), glutamic acid decarboxylase, apoptotic biomarker (caspase-3) contents, neuropathological alterations, and serum lipid profile were determined. The results show that *Cnidoscolus aconitifolius* post-treatment significantly attenuated neurological, motor, and memory deficits in rats after reperfusion in the BCCAO model. Furthermore, *Cnidoscolus aconitifolius* post-treatment significantly reduced brain oxidative stress biomarkers, myeloperoxidase, pro-inflammatory cytokines, NF-κB, and caspase-3, while normalizing serum lipid profiles in rats with ischemic stroke. Cnidoscolus aconitifolius administration reduces brain damage, significantly enhances neuronal viability, increases neuronal antioxidant defenses, and increases glutamic acid decarboxylase activity in ischemic rats after reperfusion. The findings demonstrated that post-treatment with *Cnidoscolus aconitifolius* enhances functional recovery in ischemic rats after reperfusion. This recovery is driven by the activation of neuroprotective pathways, as evidenced by reduced brain damage, increased glutamic acid decarboxylase activity, and enhanced antioxidant defenses.

Introduction

Stroke is a prevalent chronic neurological disease and leading cause of death as well as long-term disability globally [1]. Clinically known as a cerebrovascular accident, it is divided into two main subtypes: hemorrhagic stroke, which is caused by a ruptured vessel, and ischemic stroke (IS). IS is the most common subtype, resulting from occlusion of cerebral vessels-particularly the carotid arteries-followed by reperfusion injury, which represents a central mechanism of its pathology [1, 2]. Currently, IS is the second highest contributor to mortality as well as long-term morbidity in the world. According to the WHO, over 15 million people suffer stroke annually, with about five million deaths, while more than half of the survivors live with permanent physical disabilities [3]. Beyond physical disabilities, stroke is frequently accompanied by psychiatric comorbidities, including anxiety, depression, and cognitive deficits, which collectively contribute to its substantial global disease burden [3]. The etiology of IS includes cardio-embolism, atherosclerosis, and small-vessel diseases, though about one-third of cases remain cryptogenic, typically occurring in younger patients with relatively mild stroke [4]. Clinical data show that controlling high blood pressure, persistently elevated blood glucose levels, and hypercholesterolemia considerably lowers stroke occurrence [5]. At the pathophysiological level, cerebrovascular obstruction results in diminished oxygen supply, initiating a cascade that includes ionic imbalance, oxidative stress, apoptosis, excitotoxicity, neuroinflammation, and autophagy, leading to neuronal cell death [1, 2]. Notably, such neuronal cell death may be permanent or long-term, manifesting as cognitive impairment, cerebral palsy, motor dysfunction, or death in severe cases. Ischemia typically originates with cerebral hypoxia, which compromises neuronal integrity. While reperfusion restores blood flow to the affected region, it paradoxically accelerates secondary injury, a process termed ischemia-reperfusion injury [2, 4]. This secondary damage is primarily driven by excitotoxicity and inflammatory responses [1, 4]. Stressed neurons, in particular, can activate microglia, which further release IL-6 and TNF-α, causing the exacerbation of secondary neuronal injury [1, 4]. Current management of IS involving the use of tissue plasminogen activators-such as alteplase, reteplase, and tenecteplase-is quite limited in scope, as these drugs cannot halt its progression [6-8]. Further, serious adverse effects, including bleeding and a high tendency to cause intracerebral hemorrhage, have compromised their clinical efficacy. In addition, the strict need to administer them as quickly as possible after a stroke episode makes treatment almost impracticable for patients in most African settings [9, 10]. Studies have shown that dietary polyphenols can mitigate the adverse consequences of stroke [9, 11]. Additionally, epidemiological evidence demonstrates that consistent eating of fruits as well as vegetables, rich in polyphenol constituents, reduces stroke risk [9, 11-13]. Therefore, there is an increasing need to evaluate the efficacy of plant-based remedies that will both restore blood flow in the ischemic brain region and elicit neuro-restoration after IS.

Cnidoscolus aconitifolius (Mill.) I.M. Johnst (Euphorbiaceae), commonly called spinach tree, is a perennial shrub indigenous to the Yucatán Peninsula, Mexico [14]. It is known as Efo Iyana Ipaja and Hospital Too Far in Southwestern and South-South regions of Nigeria, respectively. Many people consume *Cnidoscolus aconitifolius* (*CA*) as a green vegetable in salads and soups, and its leaves are very rich in vitamins and antioxidant molecules [14]. The leaves are utilized as diuretics, circulation enhancers, and for maintenance of nervous health in traditional medicine [14]. Its anti-diabetic, hepatoprotective, antimicrobial, and anti-rheumatic properties have been reported [15, 16]. A variety of bioactive substances found in *CA* leaves include chlorogenic, vanillic, protocatechuic, caftaric, 4-hydroxybenzoic, caffeic, p-coumaric, as well as ferulic acid, which have exhibited antioxidant, anti-mutagenic, cardioprotective, and anticancer properties [14]. In addition, HPLC analysis also confirmed the presence of bioactive compounds including kaempferol, quercetin, amentoflavone, hesperidin, and protocatechuic acid in *CA*, which have been found to reduce the risks of cardiovascular diseases [14, 17]. Neurobehavioral and biochemical studies have shown that *CA* enhances cognition by reducing acetylcholine-

sterase activity [18]. Notably, its potential benefit in IS stems from its long-term use in ethnomedicine in enhancing circulation and reducing high cholesterol alongside atherosclerosis by the Maya natives in Mexico and Central America [19]. Traditional healers frequently prescribe leaf infusions or teas to treat systemic cardiovascular problems associated with thrombosis and impaired blood flow [19]. It is worthy of note that thrombosis and impaired blood flow are major pathological hallmarks of IS [2, 9]. Nevertheless, no detailed studies have looked at its possible benefits in post-IS disorder. Therefore, this study was designed to assess its neuroprotective properties in male Wistar rats subjected to ischemia-reperfusion (IR) injury.

Materials and methods

Source of plant and extraction: *CA* leaves collected on 22 April 2022 from a vegetable garden, Orisunbare Apata, Ibadan, Nigeria, were identified at the Forestry Research Institute of Nigeria, Ibadan, with herbarium number FHI 113365. The powdered dried leaves material (700 g) was soaked in distilled water (5.0 L) for 72 hrs. The resulting solution was filtered and concentrated using a rotary evaporator. Distilled water was utilized to reconstitute the residue known as *CA* at the concentrations needed for the investigation. The doses of 50, 100, and 200 mg/kg of *CA* were selected based on previous studies [20].

Chemicals: Sigma-Aldrich (St. Louis, USA) supplied the thiobarbituric acid, 5,5'-dithiobis(2-nitrobenzoic acid), ketamine HCl, and adrenaline. Elabscience (Wuhan, China) supplied the ELISA kits-NF-κB and caspase-3, while BioLegend (San Diego, USA) provided the kits for TNF-α and IL-6.

Experimental animals: Forty male Wistar rats (200-250 g, 10-12 weeks old) were procured from the Animal Facility, University of Ibadan, Nigeria. They were kept in cages under standard laboratory conditions of a 12-hrs light/dark cycle and 50-60% relative humidity. With the exception of a 12-hr fast before surgery, food and water were freely available to them. The rats were acclimatized for one week before commencement of the study. The experimental protocols were approved by the Animal Care and Use Research Ethics Committee, University of Ibadan, Nigeria (UI-ACUREC/097-1023/11) and were conducted in accordance with the ethical principles established by the National Institutes of Health Guide (NIH) for the Care and Use of Laboratory Animals.

Bilateral common carotid artery occlusion surgical procedures: Prior to surgery, the rats were distributed into five groups (n=8) using stratified randomization. Group 1: sham control, group 2: IR control, group 3: *CA* (50 mg/kg), group 4: *CA* (100 mg/kg), and group 5: *CA* (200 mg/kg). The baseline body weight of the rats was used as the stratification factor, and a random allocation sequence was generated using a random number table. The rats in groups 2 to 5 were subjected to BCCAO IS according to the procedures earlier described [6, 21]. The rats were treated with ampicillin (100 mg/kg) for antibacterial prophylaxis. They were given an injection of diazepam (20 mg/kg, i.p.) with ketamine (80 mg/kg) for surgical anesthesia. Then, the two common carotid arteries of the rats were separated from the associated vagosympathetic trunks through a midline neck incision. This was followed by the occlusion of the common carotid arteries using a 4-0 suture for 30 min for induction of global ischemia. Thereafter, reperfusion was established following the removal of the sutures, and the structural integrity of the arteries was visually confirmed. Sixty minutes after reperfusion, the rats in group 2 (IR control) received distilled water (10 mL/kg, p.o.), while groups 3 to 5 received oral doses of *CA* (50, 100, or 200 mg/kg) once daily for seven days. The rats in group 1 (sham control) received distilled water (10 mL/kg, p.o.) daily for seven days and went through surgical procedure but their arteries were not occluded. On day seven after reperfusion, the mortality rate was recorded, and the rats (n=4-7) that recovered were subsequently assessed for neurological, motor, and memory functions by trained personnel blinded to the treatments. Thereafter, the rats (n=4) across all the groups were euthanized for brain biochemical analyses, while histomorphological studies were done using three animals per group (n=3) by trained personnel blinded to the treatments (**Figure 1**).

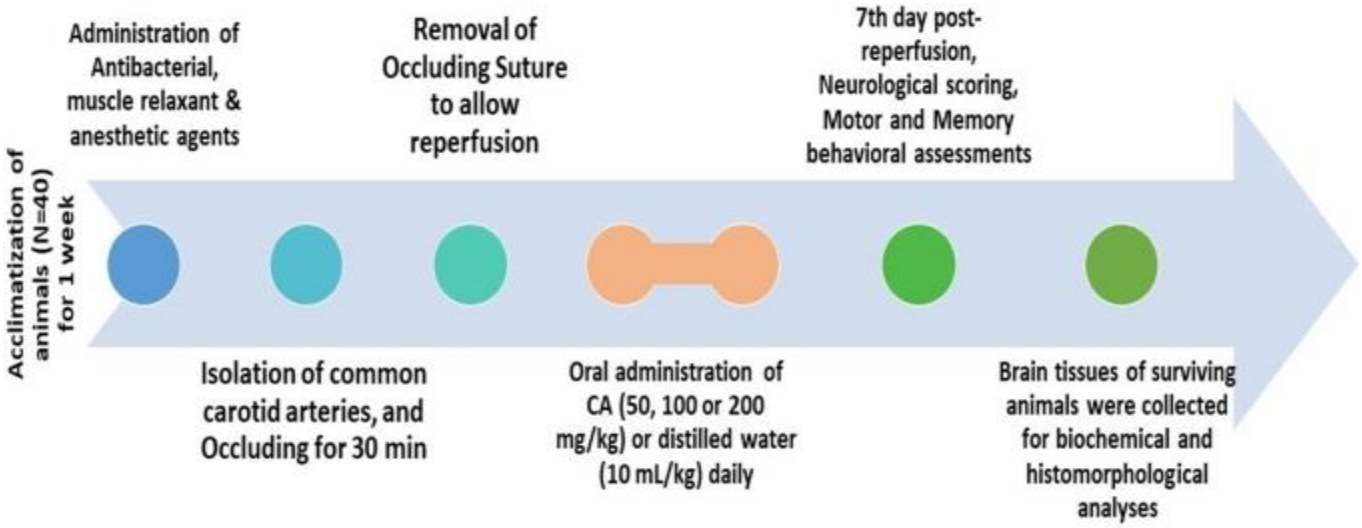

Figure 1: Exemplification of experimental protocols showing induction of ischemia, reperfusion, duration of treatments and behavioral, biochemical, and histomorphological analyses

Neurological function assessment: Neurological functions were evaluated on day seven after reperfusion using nine-point neurological scores, with the assessor blinded to treatments [6, 21]. Briefly, body torsion was assessed by suspending each rat by its tail 10 cm above the ground for five sec and scored from 0 to 3: 0 (normal, extending downward without twisting), 1 (showing signs of cortical lesions, swinging to the opposite side), 2 (twisting partially toward the tail), or 3 (twisting completely). Forelimb flexion was scored from 0 to 3: 0 (normal, extending forelimbs and attempting to grasp the ground), 1 (showing signs of mild injury by exhibiting contralateral forepaw bending), 2 (moderate injury exhibiting 45° flexion), or 3 (severe, showing 90° flexion) [5]. The beam-walking test utilized a thin beam (3.0 × 70cm) raised 20 cm above the bench to assess motor coordination and tetraparesis via grip ability. Strong grip (0), sporadic loss of grip (1), no grip with limbs resting on the beam (2), or no grip with limbs dangling and unable to walk along the beam (3). Combining findings from these three tests yielded a maximum cumulative score of nine points.

Spatial memory assessment: The Y-maze test was deployed for spatial memory assessment on the seventh day following reperfusion. Each rat explored the three arms of the maze for five minutes, and the number of arm entries as well as alternations was recorded [22]. An arm entry was counted each time a rat's four paws were completely inside an arm. The memory function, given as percentage alternation, was obtained through division of the number of correct alternations by the total number of arm entries minus two [22].

Test for locomotor activity: On day seven after the stroke, locomotion was tested using an open field apparatus, specifically a large plywood box with a 90 × 90 cm square base, 45 cm high walls, and a floor divided into 25 equal grids [23]. The rats were placed individually in the center, and line crossings were counted for five minutes.

Anxiety test: The elevated plus maze protocol was used for the evaluation of the anxiety-like behaviors, as earlier described [24-26]. The maze consisted of two open arms, two enclosed arms, and a central platform. The rats were separately placed at the edge of an open arm, and the number of entries into and time spent in each arm were documented over a five-minute test period. Thereafter, the Index of Open-arm Avoidance (IOAA) was calculated as ([100 - {percentage time in open arms} + {percentage entries into open arms}/2).

Blood sample collection and brain tissue preparation: Rats were anesthetized with intraperitoneal injections of ketamine (75 mg/kg) and diazepam (2.5 mg/kg), and blood samples were collected via ocular puncture into plain tubes for lipid profile estimation. The brains were harvested, rinsed with ice-cold phosphate-buffered saline, and

weighed before the right hemispheres were dissected on ice for isolation of the prefrontal cortex (PFC), striatum, and hippocampus. To isolate these regions, the brain was first divided hemispherically. The PFC was obtained by slicing the anterior tip. The striatum was dissected from the subcortical gray matter beneath the corpus callosum, and the CA1 region was isolated by unrolling the outer dorsal layer of the hippocampus. The brain portions designated for biochemical analyses were homogenized in ice-cold phosphate-buffered saline before centrifugation at 10,000 rpm for 10 min at 4°C. The other brain portions intended for neuropathological analyses were fixed in 10.0% formalin.

Serum lipid profile and atherogenic index (AI) determination: Serum lipid profile biomarkers, including triglycerides, total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-c), were estimated using a Randox kit according to the specifications of the manufacturer. Low-density lipoprotein cholesterol (LDL-c) and very-low-density lipoprotein cholesterol (VLDL-c) were calculated using the formula of Friedewald as previously reported [24]. AI was determined as previously published [24].

Assays of malondialdehyde (*MDA), nitrites, and antioxidants in rat brain regions:* The MDA content was estimated using the method of Ohkawa et al. [27] of thiobarbituric acid reactive substances and its levels reported as ηmol/mg protein. The Griess colorimetric test was employed for the determination of nitrite concentration (ηmol/mg protein), as reported [24]. As reported, the concentration of reduced glutathione (GSH) was determined as earlier reported [24]. The activities of antioxidant enzymes including catalase (U/mg protein), glutathione-S-transferase (GST), and superoxide dismutase (SOD) earlier reported [28]. The method described by Lowry et al. [29] was employed for protein content estimation.

Pro-inflammatory cytokine assays: Pro-inflammatory cytokines (TNF-α, IL-6) concentrations were measured using ELISA kits (BioLegend, San Diego, USA) in accordance with the manufacturer's guidelines. Following the incubation of biotinylated antibodies and streptavidin-peroxidase conjugate, a chromogen substrate and a stop solution were added. The absorbance was then measured at 450 nm using a microplate reader (LT4500, UK) and reported as pg/mg of protein

Glutamic acid decarboxylase (GAD) assay: The procedures highlighted by Yu et al. [30] and reported by Abbas et al. [24] were followed for the estimation of GAD activity in brain tissue supernatant of the rats. The enzymatic reaction was initiated by incubating 50.0 µL of 2X-diluted brain tissue supernatant with a reaction mixture containing 20 mM sodium acetate, 70.0 µM bromocresol, 10 mM pyridoxal-5-phosphate, and 2.0 µL of 1.0 M glutamate. The microplate reader was used to measure the increase in absorbance at 630 nm over a 5 min period. The activity was quantified using an extinction coefficient of 23700/ M/ cm and expressed as U/mg protein.

Assay for myeloperoxidase (MPO): According to Suzuki et al. [31], MPO activity was measured using 3,3′,5,5′-tetramethylbenzidine (TMB). Each brain sample (10.0 µL) was combined with 1110 µL of TMB solution (2.9 mM TMB in 14.5% DMSO and 100 mM sodium phosphate buffer, pH 5.4) and 80.0 µL of 0.75 mM H_2O_2. After incubation at 37°C for five minutes, the reaction was stopped by adding 2.0 M H_2SO_4 (50 µL). Absorbance was measured at 450 nm, and MPO activity was quantified as U/mg protein.

Assays for caspase-3 and NF-κB: ELISA kits were used for brain caspase-3 and NF-κB contents following manufacturers' protocols, and absorbance was read at 450 nm using a microplate reader (LT4500), and caspase-3 activity was quantified as U/mg protein, while NF-κB contents were expressed in pg/mL protein.

Histomorphological studies of the PFC, striatum, and hippocampus: Histomorphological analysis of the distinct brain section was done as earlier described [32]. Each brain tissue region underwent routine histological processing, including dehydration, clearing, paraffin infiltration, embedding, and sectioning. Coronal sections (5.0-6.0 µm) of the PFC, striatum, and hippocampal CA1 area were cut using a microtome and stained with

Hematoxylin and Eosin for neuropathological evaluation. Slides were inspected using a Leica DM500 digital light microscope, and photomicrographs were captured using a Leica ICC50 E digital camera coupled to a computer interface. Histomorphometric analysis was conducted using a computerized image analyzer (Apache Open Office). Neuronal viability was assessed in ten randomly selected fields from the PFC, stratum, and hippocampal CA1 region for each experimental group [32].

***Statistical analysis*:** Data are presented as mean±standard error of the mean (SEM). To compare the groups, one-way ANOVA followed by Tukey's post hoc test for parametric data was used. Non-parametric data were analyzed using the Kruskal-Wallis test followed by Dunn's post hoc test when it is applicable. The analyses were performed using GraphPad Prism (version 8) (GraphPad Software, Inc., CA, USA), and $p<0.05$ was considered statistically significant.

Results

Cnidoscolus aconitifolius post-treatment on mortality of rats after IS: **Table 1** depicts the effects of *CA* on the mortality rate of rats subjected to ischemia/reperfusion injury. Mortality was recorded in rats that underwent the BCCAO IS protocol. In contrast, no deaths were recorded in the sham group and the group treated with the lowest dose of *CA* on day seven after reperfusion. As shown in **Table 1**, higher oral doses of *CA* post-treatment reduced the mortality rate by one on day seven post-reperfusion.

Table 1: Effect of aqueous leaf extract of *Cnidoscolus aconitifolius* (*CA*) post-treatment on mortality in rats after ischemia/reperfusion episode

Group	Mortality (24 hrs)	Mortality (24 hrs)	Mortality (day 7)	Mortality (day 7)
Sham control	1/8	12.5	0/7	0
IR control	2/8	25	2/6	33.3
CA 50 mg/kg	2/8	25	0/6	0
CA 100 mg/kg	1/8	12.5	1/7	14.3
CA 200 mg/kg	2/8	25	1/6	16.7

Cnidoscolus aconitifolius post-treatment improves neurological, memory, and motor impairments in rats with IS: The effects of *CA* post-treatment on neurological score, spatial working memory, and spontaneous motor activity in rats after IR are presented in **Figure 2** and **Table 2**. There was a significant decrease in the neurological score in the rats with IS, suggesting impairment in neurological function (**Figure 2**). As shown in **Table 2**, the rats with IS demonstrated reduced alternation (%) and decreased number of line crossings, indicating impaired short-term spatial memory and reduced motor activity, respectively, when compared with sham control ($p<0.05$). Compared to IS controls, rats treated daily with *CA* for seven consecutive days following reperfusion mitigated the neurological, memory, and motor deficits, relative to sham controls ($p<0.05$).

Table 2: *Cnidoscolus aconitifolius* (*CA*) post-treatment improves neurological, memory, and motor functions in rats after an ischemia/reperfusion episode

Group	Alternation (%)	Number of line crossings
Sham control	90.0 ± 5.0	30.0 ± 2.0
IR control	25.0 ± 2.0 $^{\#}$	12.0 ± 1.4 $^{\#}$
CA 50 mg/kg	70.0 ± 5.0 *	18.0 ± 1.0 *
CA 100 mg/kg	60.0 ± 4.0 *	20.0 ± 1.2 *
CA 200 mg/kg	50.0 ± 4.0 *	24.0 ± 1.3 *

Values are mean ± SEM (n=4-7). $^{\#}$ $p < 0.05$ compared with sham control; * $p< 0.05$ compared with IR, using one -way ANOVA followed by Tukey's multiple comparison test

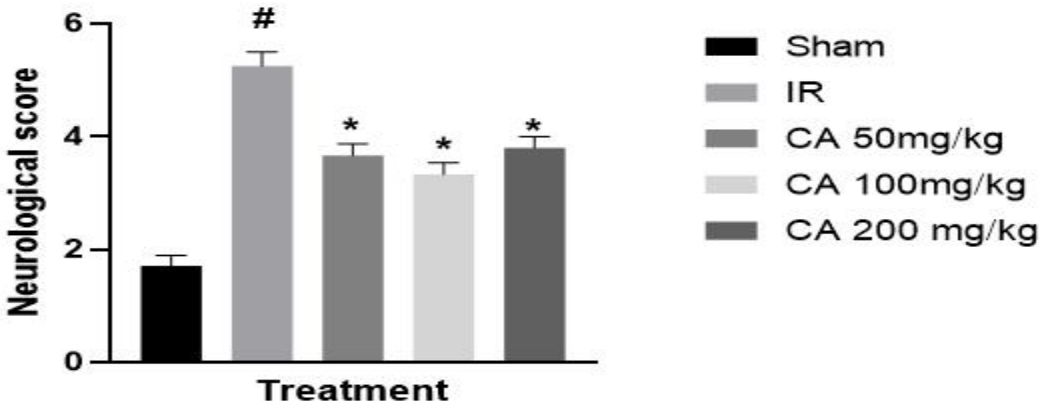

Figure 2*:* *Cnidoscolus aconitifolius* (*CA*) post-treatment reduces neurological score in rats after ischemia-reperfusion.

Values represent 4-7 rats/group. # $p < 0.05$ compared with sham control; * $p < 0.05$ compared with IR, using the Kruskal-Wallis test followed by Dunn's multiple comparison test.

Cnidoscolus aconitifolius p*ost-treatment decreases anxiety-like behavior in ischemic rats:* The effects of *CA* post-treatment on anxiety-like behaviors in rats following BCCAO and reperfusion are presented in **Figure 3**. As depicted in **Figure 3**, rats with IS exhibited anxiety-like features reflected by reduced duration of time spent in the open arm of the elevated plus maze and increased avoidance index, relative to the sham groups. Conversely, seven consecutive days of CA post-treatment significantly increased the time spent in the open arms of the elevated plus maze and reduced the avoidance index compared to the IR group.

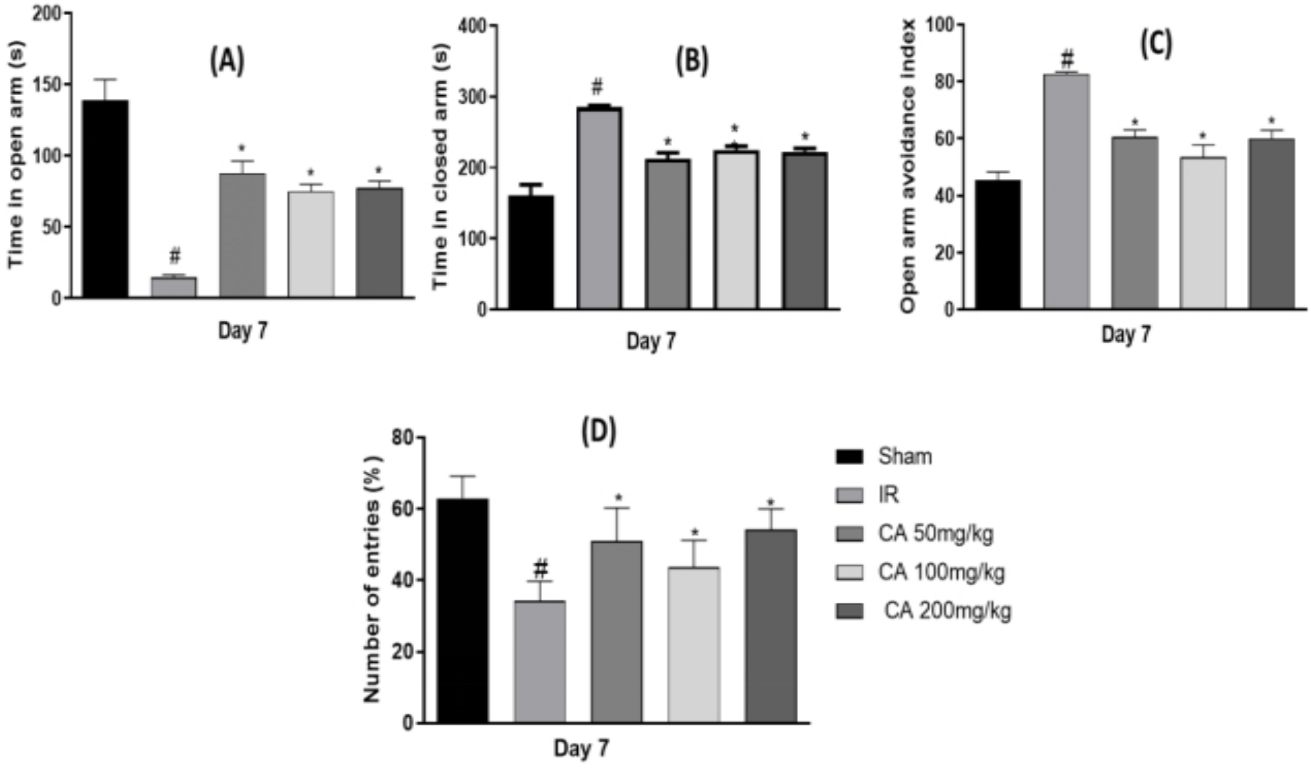

Figure 3: *Cnidoscolus aconitifolius* (*CA*) post-treatment reduces anxiety-like characteristics: Time in open arm (A), Time in closed arm (B), Arm avoidance index (C), and Number of entries in open arm (D) in rats with ischemic stroke.

Values are mean±SEM (n=4-7 rats/group). # $p < 0.05$ compared with sham control; * $p < 0.05$ compared with IR using one-way ANOVA followed by Tukey's multiple comparison test.

Effects of post-treatment with CA on lipid profile in rats after IS: When compared to the sham controls, IS dramatically altered the rats' serum lipid profiles, as illustrated in **Table 3**. This was evidenced by a significant elevation of triglycerides, total cholesterol, LDL cholesterol, and AI, alongside a decrease in HDL cholesterol ($p < 0.05$). Conversely, *CA* (50, 100, and 200 mg/kg) attenuated these lipid dysregulations. It restored triglyceride, total cholesterol, and HDL cholesterol levels toward control values, while lowering LDL cholesterol and significantly reducing the AI relative to the IR groups.

Table 3: Post-treatment with *Cnidoscolus aconitifolius* (*CA*) modulates lipid profile in rats after ischemic stroke

Groups	Triglyceride	TC	HDL	LDL	AI
Sham control	131.0 ± 1.37	88.12 ± 1.06	40.23 ± 0.89	21.57 ± 0.10	1.19 ± 0.04
IR group	173.3 ± 3.51 *	104.2 ± 2.89 *	34.31 ± 0.93 *	35.20 ± 1.21 *	2.04 ± 0.09 *
CA 50 mg/kg	131.0 ± 1.37 *	88.90 ± 0.91 *	38.70 ± 0.82 *	23.80 ± 0.85 *	1.30 ± 0.04 *
CA 100 mg/kg	137.0 ± 1.36 *	92.00 ± 2.34 *	38.40 ± 1.09 *	28.70 ± 1.13 *	1.40 ± 0.07 *
CA 200 mg/kg	129.0 ± 2.02 *	96.60 ± 0.78 *	40.90 ± 0.64 *	29.80 ± 0.44 *	1.36 ± 0.03 *

Values are mean±SEM (n=4 rats/group). # $p < 0.05$ compared with sham control; * $p < 0.05$ compared with IR using one-way ANOVA followed by Tukey's multiple comparison test.
TC - total cholesterol, HDL - high-density lipoprotein, LDL - low-density lipoprotein, AI - atherogenic index

Post-treatment with CA modulates pro-oxidant and antioxidant contents in rats with IS: The effects of *CA* on pro-oxidant and antioxidant levels within the PFC, striatum, and hippocampal CA1 regions of rats subjected to the BCCAO model are depicted in **Figures 4, 5,** and **6**. Compared to the sham controls, reperfusion in ischemic rats led to a significant increase in brain pro-oxidant markers-specifically MDA and nitrites- alongside a concomitant depletion of antioxidant defenses, including SOD, catalase, GSH, and GST. Conversely, *CA* post-treatment dramatically ($p<0.05$) decreased quantities of pro-oxidant molecules while restoring antioxidant defenses in specific regions of the brain of ischemic rats following reperfusion.

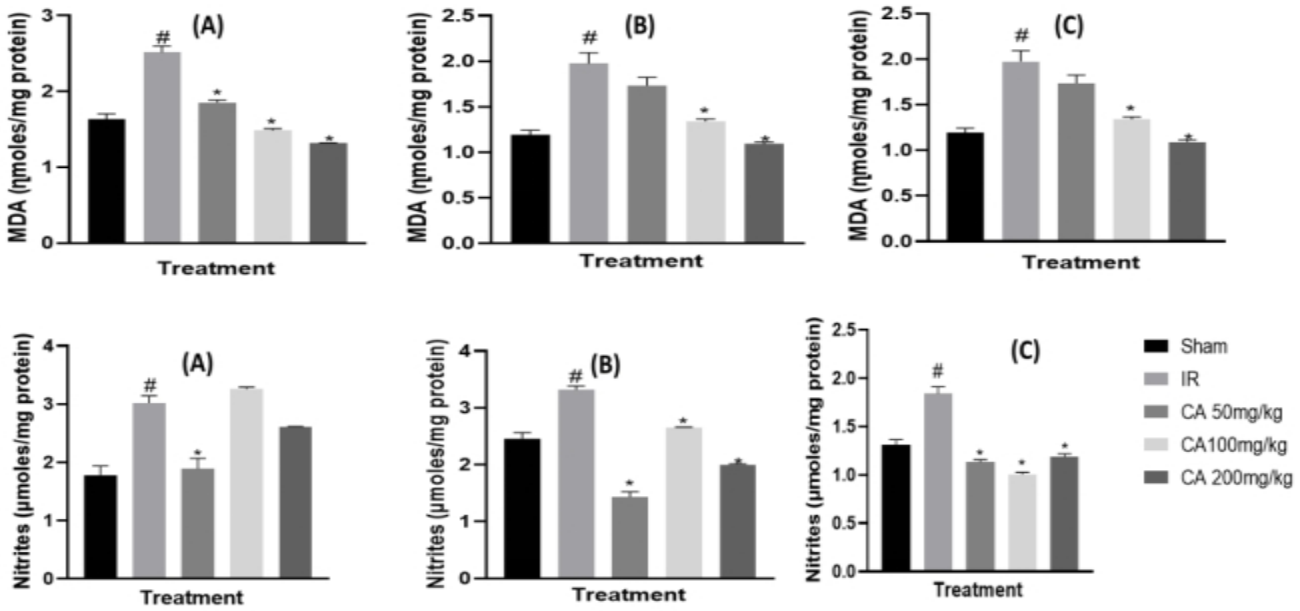

Figure 4: *Cnidoscolus aconitifolius* (*CA*) post-treatment decreases MDA and nitrite contents in rats' striatum (A), prefrontal cortex (B), and hippocampus (C) following ischemia/reperfusion.

Values are mean±SEM (n=4 rats/group). # $p < 0.05$ compared with sham control; * $p < 0.05$ compared with IR using one-way ANOVA followed by Tukey's multiple comparison test.

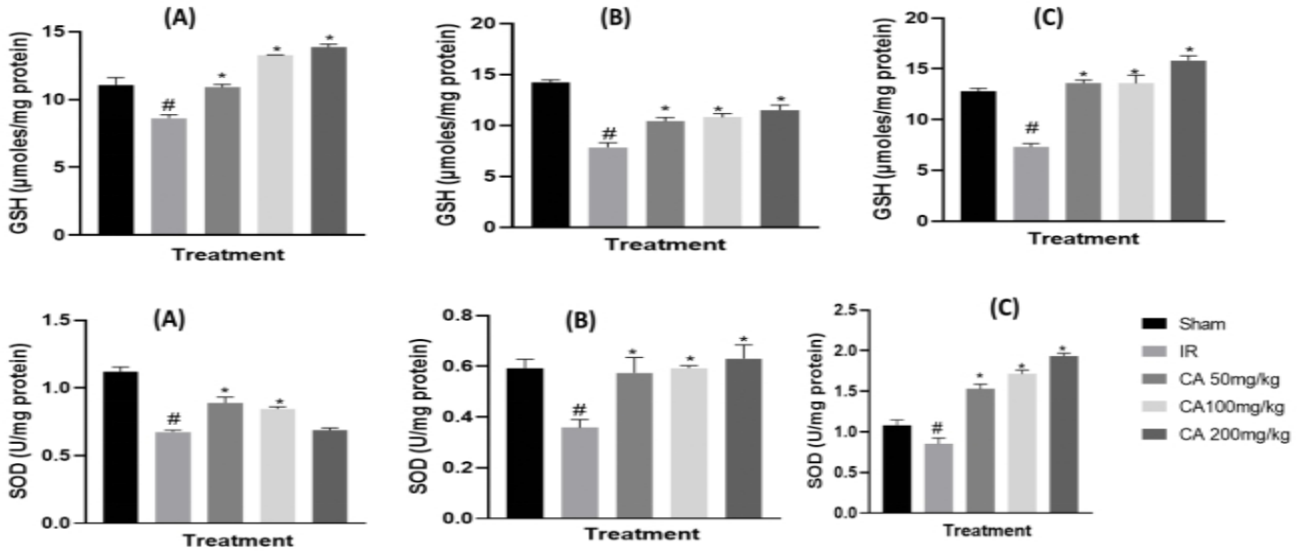

Figure 5: *Cnidoscolus aconitifolius* (*CA*) post-treatment increases GSH and SOD contents in rats' striatum (A), prefrontal cortex (B), and hippocampus (C) following ischemia/reperfusion.

Values are mean±SEM (n=4 rats/group). # $p < 0.05$ compared with sham control; * $p< 0.05$ compared with IR using one-way ANOVA followed by Tukey's multiple comparison test.

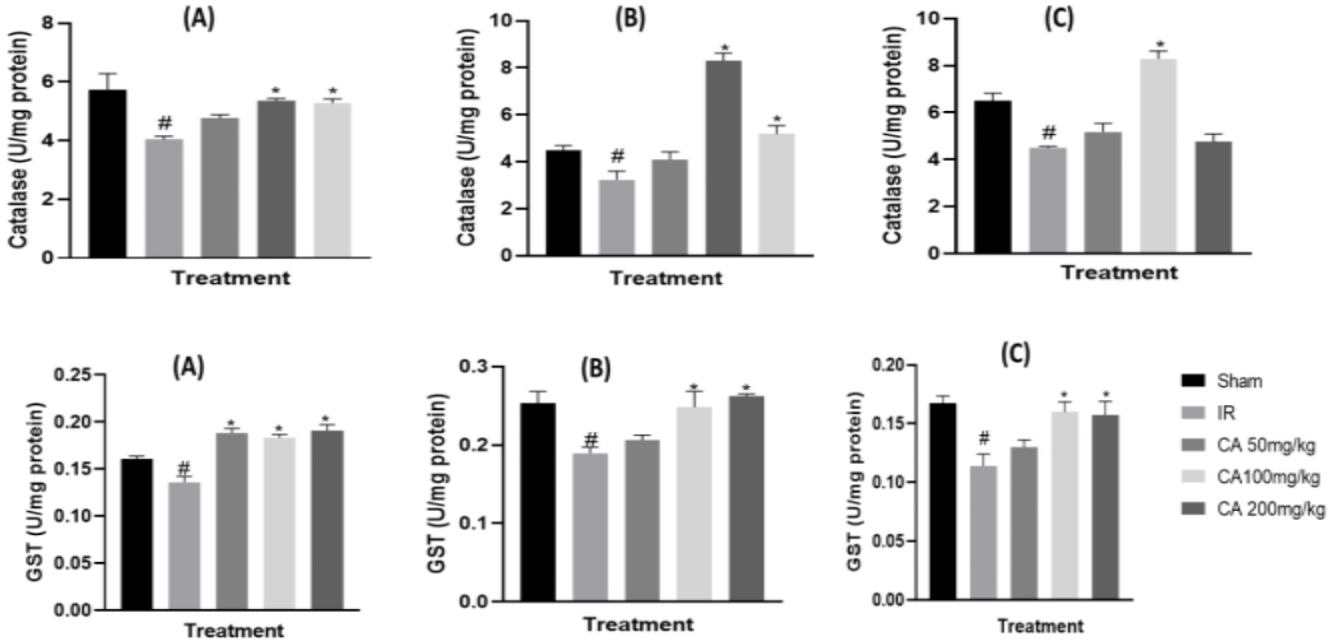

Figure 6: *Cnidoscolus aconitifolius* (*CA*) post-treatment increases catalase and GST activities in the rats' striatum (A), prefrontal cortex (B), and hippocampus (C) after ischemia-reperfusion.

Values are mean±SEM (n=4 rats/group). # $p < 0.05$ compared with sham control; * $p< 0.05$ compared with IR using one-way ANOVA followed by Tukey's multiple comparison test.

Cnidoscolus aconitifolius decreases brain regions' pro-inflammatory cytokines in rats after IS: **Figure 7** shows the effects of *CA* post-treatment on pro-inflammatory cytokine levels in the rat PFC, striatum, as well as hippocampal CA1 regions following reperfusion. Compared to the sham controls, rats subjected to the BCCAO model exhibited significantly higher levels of IL-6 and TNF-α across these distinct brain regions. In contrast, *CA* (100 and 200 mg/kg) produced a marked decrease in pro-inflammatory cytokines in the brain areas of rats in comparison to IR groups ($p<0.05$).

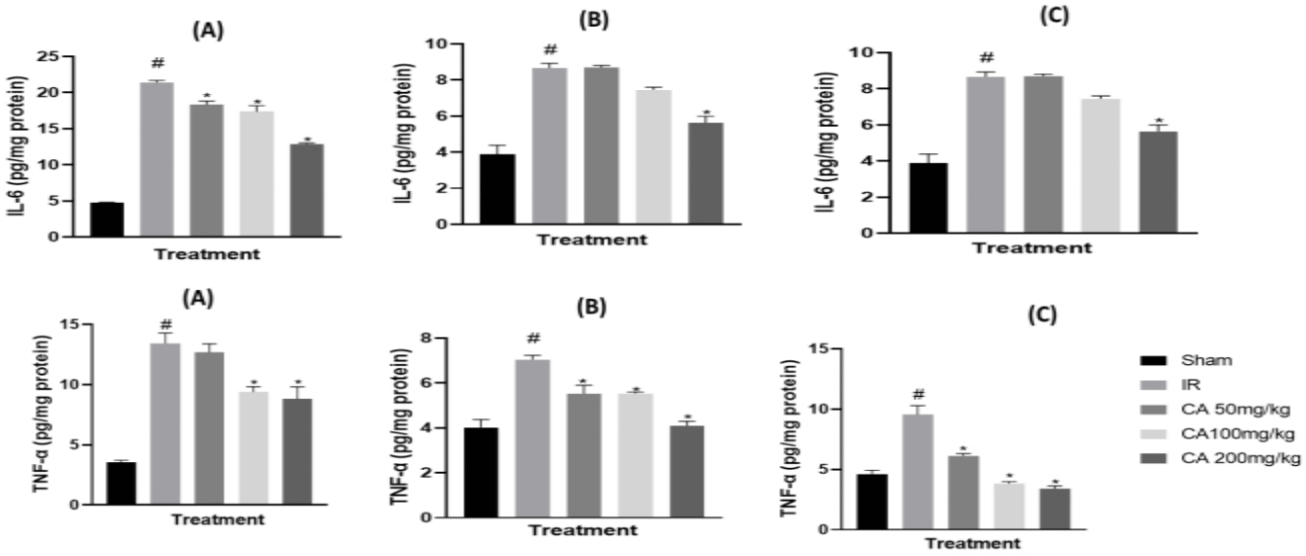

Figure 7: *Cnidoscolus aconitifolius* (*CA*) post-treatment decreases pro-inflammatory cytokine concentrations in the rats' striatum (A), prefrontal cortex (B), and hippocampus (C) following ischemia-reperfusion.

Values are mean±SEM (n=4 rats/group). # p<0.05 compared with sham control; * p<0.05 compared with IR using one-way ANOVA followed by Tukey's multiple comparison test.

Post-treatment with CA on GAD and NF-κB levels *in rats' brain regions after IS:* Compared with the sham controls, rats subjected to the BCCAO IS model showed significant decreases in GAD activity and elevated NF-κB levels in the PFC, striatum, and hippocampal CA1 regions. Notably, *CA* post-treatment (50, 100, and 200 mg/kg) significantly decreased NF-κB contents in these specific brain areas of ischemic rats and restored GAD activity in the PFC and striatum compared with the IR groups (**Figure 8**).

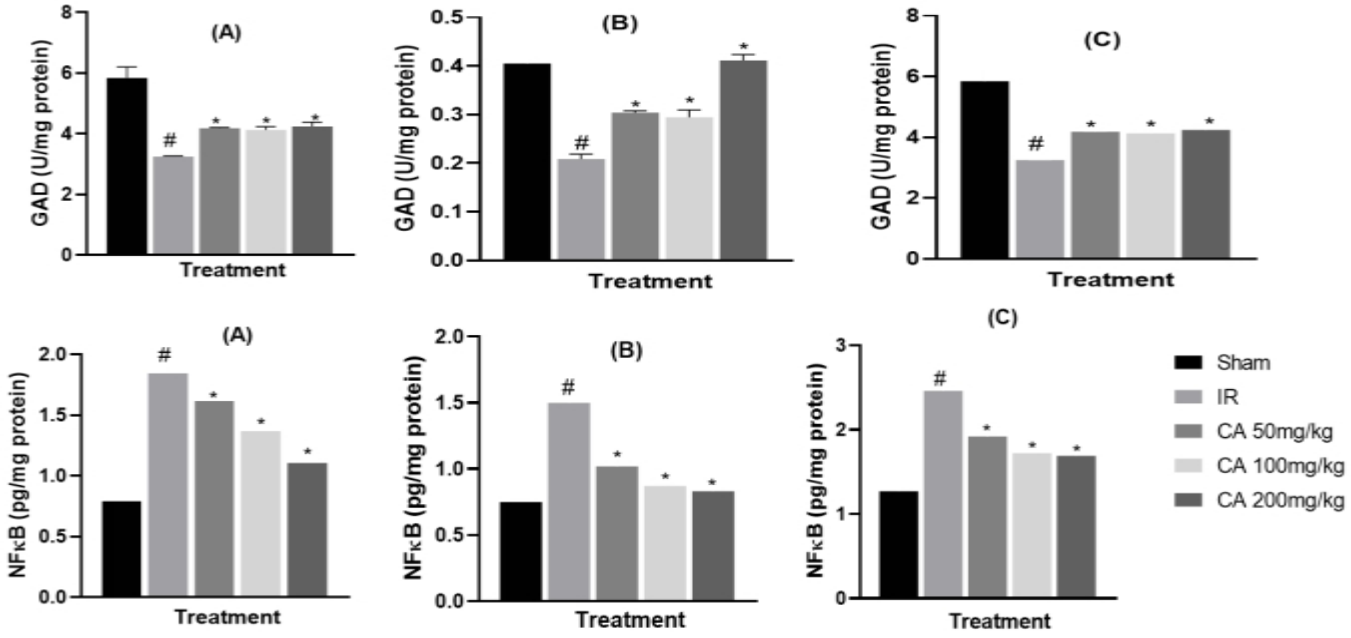

Figure 8: *Cnidoscolus aconitifolius* (*CA*) post-treatment reduces NF-kB and increases GAD contents in the rats' striatum (A), prefrontal cortex (B), and hippocampus (C) after ischemic stroke.

Values are mean±SEM (n=4 rats/group). # p < 0.05 compared with sham control; * p<0.05 compared with IR using one-way ANOVA followed by Tukey's multiple comparison test.

Cnidoscolus aconitifolius administration reduces MPO and caspase-3 contents *in rats' brain regions after IS*: As shown in **Figure 9**, rats exposed to the BCCAO-induced global IS model exhibited significant increases in MPO and caspase-3 levels, relative to sham controls. Conversely, post-ischemic treatment with *CA* (50, 100, and 200 mg/kg) significantly lowered MPO and caspase-3 contents in specific brain regions, relative to IR groups.

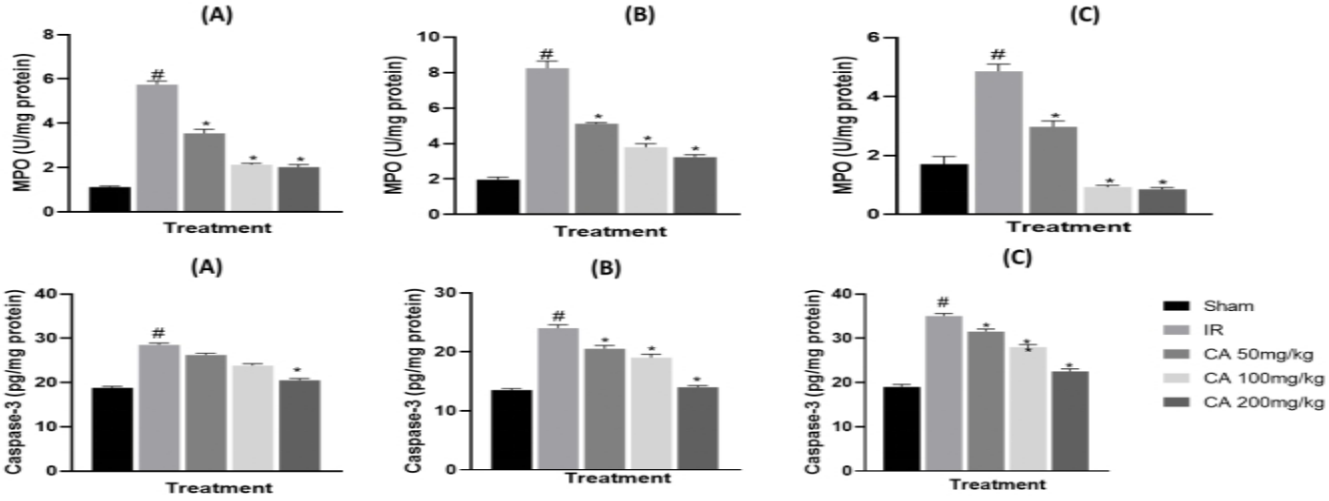

Figure 9: *Cnidoscolus aconitifolius* (*CA*) post-treatment decreases myeloperoxidase (MPO) and caspase-3 in the striatum (A), prefrontal cortex (B), and hippocampus (C) of rats with ischemic stroke.

Values are mean±SEM (n=4 rats/group). # $p < 0.05$ compared with sham control; * $p<0.05$ compared with IR using one-way ANOVA followed by Tukey's multiple comparison test.

Cnidoscolus aconitifolius post-treatment preserves neuronal morphology of rats after IS: The effects of seven consecutive days of *CA* post-treatment on the histopathological changes and neuronal viability of the PFC, striatum, and hippocampal CA1 tissues of rats stained with H & E are presented in **Figures 10** and **11**. The IS episode induced marked structural distortions, including neuronal shrinkage, loss of cellular integrity, and reduced neuronal cell count within the PFC, striatum, and hippocampus. Conversely, *CA* post-treatment preserved neuronal integrity across these brain regions and significantly increased the number of viable neuronal cells (**Figures 10** and **11**).

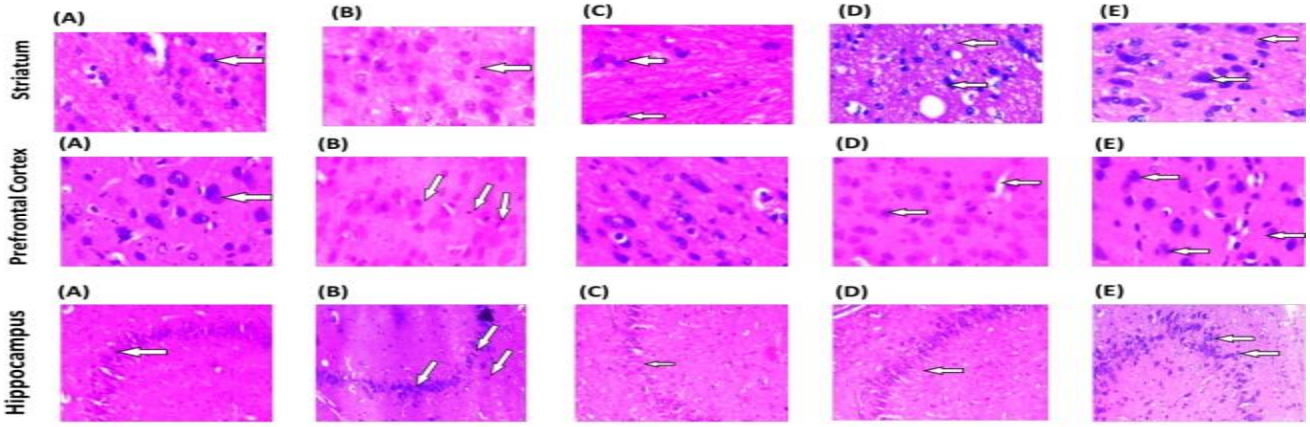

Figure 10: Photomicrographs showing the effects of *Cnidoscolus aconitifolius* on H&E-stained brain regions of rats subjected to ischemia/reperfusion.

Plate A (Sham-control) shows normal neurons with hyperchromatic nuclei; B (Ischemia-reperfusion) depicts neuropil with bluish-stained/eosinophilic neurons, angulated cells, and perivascular edema; C (*CA*- 50 mg/kg), D (*CA*-100 mg/kg, and E (*CA*- 200 mg/kg) show some normal neurons with intact cells and some moderate diffuse pyknotic neurons. H&E-stained ×400.

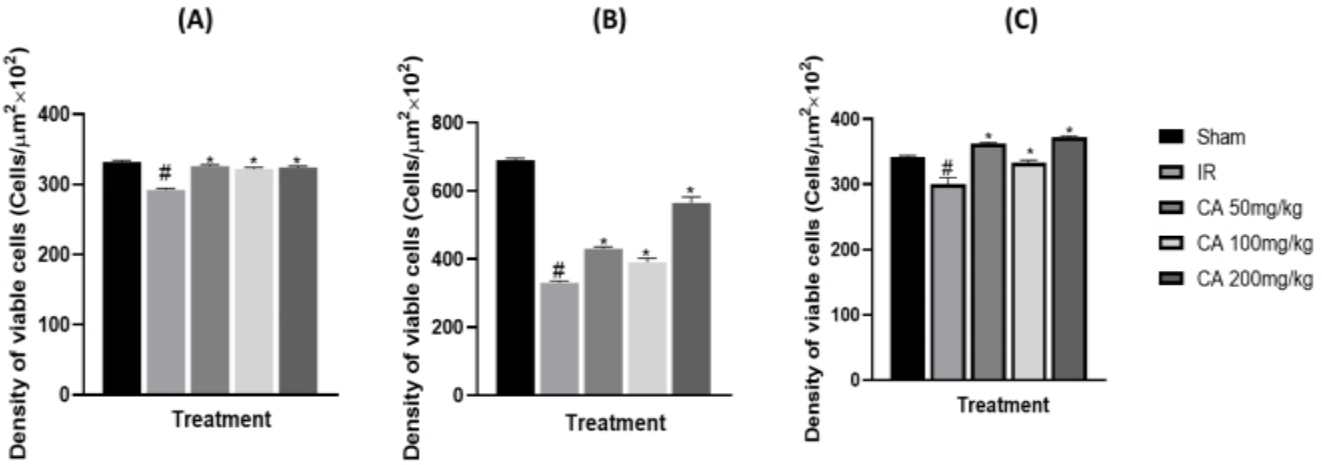

Figure 11: *Cnidoscolus aconitifolius* (*CA*) post-treatment increases viable cell count in the striatum (A), prefrontal cortex (B), and hippocampus (C) of rats following ischemia-reperfusion.

Values are mean±SEM (n = 3 rats/group). # $p < 0.05$ compared with sham control; * $p < 0.05$ compared with IR using one-way ANOVA followed by Tukey's multiple comparison test.

Discussion

Most preclinical studies of IS primarily focus on acute outcomes after reperfusion, largely evaluating infarct sizes and neurological scores as indicators of acute functional motor impairments at 24 hours [33]. Although early neurological and neuropathological outcomes are valuable, they have significant limitations. These include an inability to detect long-term impairments, functional recovery, meaningful therapeutic efficacy, or the potential impact of rehabilitation [33, 34]. Consequently, assessing post-stroke neurobehavioral phenotypes-including motor function, anxiety, and cognitive characteristics-is regarded as a more comprehensive strategy for evaluating novel therapeutics in preclinical IS models. In-depth analysis of functional recovery after brain ischemic injury offers a better framework for translating preclinical data to clinical applications and assessing new treatment strategies [33]. In this study, we used various behavioral tasks to assess the effects of seven consecutive days of *CA* treatment on the functional recovery of rats following ischemia/reperfusion. Our findings demonstrate that rats exhibited neurological deficits and impaired locomotion after the IS episode, both of which were mitigated by *CA* post-treatment. Detailed analysis of the behavioral tasks further revealed that daily treatment of rats with *CA* for seven days ameliorated the anxiety-like behaviors and cognitive impairments induced by ischemia/reperfusion. Furthermore, the data showed that daily treatment with *CA* improved the lipid profile and reduced the AI following IS. The present findings demonstrated that *CA* reduces oxidant species, pro-inflammatory cytokines, and NF-κB immunopositive cells, while enhancing the antioxidant profile and GAD activity in rats recovering from stroke. Histopathological analyses showed that *CA* treatment protected the neuronal cytoarchitecture of the PFC, striatum, and hippocampus, and increased the viable neuronal count in these brain sections after the ischemia/reperfusion insult. Notably, the ability of *CA* post-treatment to exert neuroplastic effects and enhance functional recovery in rats following reperfusion suggests its potential benefits in post-stroke conditions through multiple mechanisms involving the reduction of oxidative and inflammatory insults, alongside the stimulation of antioxidant and GAD-mediated neuroprotective pathways.

Earlier reports have shown that acute neuroprotection remains a huge challenge in clinical settings, arising from delays between stroke presentation and treatment administration [33]. Accordingly, assessment of post-stroke neurobehavioral phenotypes, including motor functions, anxiety, and cognitive characteristics, is regarded as a comprehensive strategy for the evaluation of novel therapeutics in preclinical models of IS. Thus, we examined

the effects of daily treatments of *CA* for seven days on functional deficits in addition to neurological impairments in rats after ischemic-reperfusion as ideal predictors of post-stroke functional recovery. The current data revealed that *CA* attenuated the neurological deficits, locomotion impairment, memory decline, and anxiety-like features in rats after ischemia/reperfusion. Previous studies have also shown that ischemia-reperfusion impairs functional recovery in rats [33, 34], which mirrors clinical features frequently seen in IS patients [35]. Notably, the majority of stroke survivors are permanently disabled because of various neurological and functional deficits, including loss of motor skills, postural instability, and impaired locomotion [33]. Motor impairments arising from IS limit patients' mobility, daily activities, and ability to return to work. Additionally, this raises the socioeconomic burden of IS and lowers the quality of life for patients [36]. Significant motor disorders are common among stroke survivors and can last for months or even years after the initial stroke episode [37]. In addition, stroke survivors are known to exhibit mood disorders and impaired cognitive functions, which are key determinants of their quality of life while also increasing the risk of recurrent stroke episodes. Research shows that stroke survivors experience cognitive impairment and mood disorders [38, 39]. *CA* post-treatment significantly decreased neurological score, suggesting improvement in functional recovery after ischemia/reperfusion, which further highlights its neuroprotective potential [40]. However, the reason for the decrease in memory improvement at higher *CA* dosages requires further investigation.

Dyslipidemia is a major risk factor for IS, which has been shown to worsen brain damage and hinder recovery [41]. After a stroke, there is frequently a pathological change in lipid metabolism, which includes decreased cardioprotective high-density lipoprotein cholesterol (HDL-C) and increased triglycerides, total cholesterol, and low-density lipoprotein cholesterol (LDL-C). According to Tuwar et al. [41], this pro-atherogenic condition is linked to poor outcomes and subsequent complications in stroke patients. Therefore, we employed the BCCAO rat model of IS to examine the effects of *CA* post-treatment on the serum lipid profile. The extract demonstrated potential beneficial effects by increasing HDL-C concentration while lowering LDL-C and the AI. These findings align with previous studies showing that CA exerts positive modulatory effects on serum lipid dysregulation [42]. Widespread functional deficits and long-term disabilities arising from IS are largely associated with the stimulation of oxidative and inflammatory pathways, which orchestrate the gradual degeneration of several neuronal circuits [1, 2]. Clinically, these injuries can manifest as death, abnormal movements (such as tremors and spasticity), and loss of motor skills (including motor incoordination and postural instability), cognitive dysfunction, anxiety, and depression [4, 37]. Globally, stroke ranks as the second leading contributor to death and the third leading cause of acquired disabilities [4]. The high mortality and morbidity associated with IS are mainly due to oxidative stress, neuroinflammation, and neuronal degeneration following cerebral hypoperfusion [1, 2, 4]. Preclinical studies have documented mortality in various models of IS, including bilateral common carotid artery occlusion (BCCAO) [43, 44]. Mortality rates and neurological outcomes in rodent stroke models are influenced by multiple factors, including surgical technique, environmental variation, surgical duration, and choice of anesthetic agents [42, 44]. Surgical complications and adverse anesthetic effects contribute substantially to mortality and experimental outcomes in these animals [44, 45]. To account for these confounding factors, rats in the sham group undergo identical surgical procedures and anesthetic exposure without actual vessel occlusion. In this study, we observed mortality in rats subjected to ischemia/reperfusion. However, ascribing these deaths exclusively to stroke is difficult without addressing the confounding factors like surgical complications and anesthetic adverse effects, which may limit the translational value of these findings. Notably, *CA* treatment-particularly at the lowest dose-reduced the mortality rate, indicating its potential to enhance survival. The protective effect of *CA* against mortality may stem from its ability to reduce oxidative damage, lower inflammation, and improve circulation. However, the specific mechanisms underlying the protective effects of *CA* at the lowest dose against IS-induced mortality, as well as its clinical relevance, require further investigation.

The brain cells are most vulnerable to oxidative damage, which is followed by the activation of pro-inflammatory cytokines, making oxidative stress and neuroinflammation significant elements of IS pathophysiology [45]. This susceptibility is associated with high peroxidizable lipids, reduced antioxidants, high oxygen demand, and increased iron levels. Oxidative stress-mediated excitotoxicity subsequently triggers post-ischemic inflammation [46]. Notably, reactive oxygen and nitrogen species (RONS) are known to stimulate the NF-κB signaling pathway, resulting in an increase in the expression of pro-inflammatory cytokines (TNF-α and IL-6) [47]. This process activates endothelial and resident glial cells, which recruit peripheral immune cells and further intensify inflammatory and oxidative injuries by releasing additional reactive species and proteolytic enzymes [9, 47]. Additionally, reperfusion injury following the restoration of blood flow contributes substantially to neuronal damage via a massive release of ROS [48]. Mechanistically, the initial burst of RONS and MPO during BCCAO-induced ischemia and reperfusion acts as a potent upstream driver of NF-κB activation and promotes its translocation into the nucleus. Once in the nucleus, NF-κB upregulates the transcription of pro-inflammatory cytokines, creating a continuous, self-sustaining loop of neuroinflammation and oxidative stress. Our investigation demonstrated that the BCCAO global IS model depletes endogenous antioxidant defenses and elevates pro-oxidants (MDA, nitrites, and MPO) in specific rat brain regions. These outcomes align with earlier studies revealing that oxidative stress is crucial to IS-induced neurological deficits. In line with previous research, BCCAO-exposed rats also displayed elevated TNF-α, IL-6, and NF-κB brain contents [6]. Notably, MPO is a potent oxidant molecule that plays a vital role in IS pathophysiology; its pro-inflammatory and pro-oxidative properties ultimately exacerbate brain injury [49]. Indeed, serum MPO levels are linked to stroke prognosis, serving as a predictor of severity and outcomes in both intracerebral hemorrhage and IS [50]. Conversely, CA post-treatment reduced these pro-oxidant molecules while bolstering antioxidant defenses (GSH, SOD, GST, and catalase) in discrete brain regions. Furthermore, CA lowered NF-κB immunopositive cells, as well as IL-6 and TNF-α concentrations, confirming earlier reports of its antioxidant and anti-inflammatory properties [17-19]. Beyond merely reducing individual markers, the neuroprotective efficacy of CA stems from its capacity to interrupt the self-amplifying cycle linking oxidative stress, inflammation, and GABAergic dysfunction.

This neuroinflammatory/oxidative loop directly intersects with and destabilizes the GABAergic system. The enzyme GAD regulates the rate-limiting step in the synthesis of GABA, the primary inhibitory neurotransmitter required to counteract ischemic excitotoxic cell death [51]. However, GAD is highly sensitive to the cellular microenvironment. Elevated local concentrations of TNF-α and IL-6 have been shown to transcriptionally suppress GAD65/67 expression, while excessive RONS directly oxidize the functional sulfhydryl groups on the GAD enzyme, severely impairing its catalytic activity [51, 52]. This mechanistic inhibition explains the marked reduction in GAD activity observed in our BCCAO model. When GAD activity drops, the brain's capacity to synthesize GABA is compromised, removing the crucial inhibitory check on glutamatergic signaling. Unchecked glutamate accumulation leads to hyperactivation of NMDA receptors, an excessive influx of intracellular calcium, and subsequent mitochondrial calcium overload. This excitotoxic state further accelerates the intracellular production of mitochondrial ROS, completing a destructive reciprocal loop where GABAergic failure exacerbates oxidative/inflammatory pathways [51, 52]. By attenuating MDA and suppressing TNF-alpha, *CA* effectively mitigates this reciprocal reinforcement between oxidative damage and NF-kB activation while indirectly restoring homeostatic control over the GAD/GABA axis. Programmed cell death in response to ischemic injury is initiated through the activation of caspases, a family of proteases that mediate neuronal cell death. Consistent with this, various studies have highlighted that ischemic injury is a critical trigger for the upregulation of apoptotic genes, leading to extensive neural apoptosis, with caspase-3 acting as a central executioner and a key target for therapeutic intervention [52-54]. Dade et al. [52] reported that BCCAO-induced IS markedly increased caspase-3 expression in the rat hippocampus, providing clear evidence of apoptotic pathway activation and its role in

neuronal cell death. Rats subjected to BCCAO exhibited histomorphological alterations in H&E-stained neurons within the striatum, PFC, and hippocampus, aligning with the wide range of neurological consequences seen in post-stroke episodes [55]. Furthermore, our data revealed a marked decrease in viable neuronal counts across these discrete brain regions seven days after the IS, supporting the earlier findings of Sarkaki et al. [56]. Consequently, the *CA*-mediated elevation of GAD activity observed in our study is not merely a localized neurochemical shift, but a systemic break in the reciprocal cross- talk between neuroinflammation, oxidative stress, and excitotoxicity. This comprehensive interruption of pathway cross-talk preserves GABAergic tone, halts calcium-dependent mitochondrial failure, and ultimately prevents the activation of executioner caspase-3, leading to the preservation of viable neuronal cells observed in the striatum, PFC, and hippocampus. Emerging evidence has shown that plant-derived bioactive compounds targeting multiple pathological pathways implicated in stroke-such as oxidative stress, inflammation, and apoptosis-hold promise as a therapeutic strategy for post-stroke recovery [8, 9, 11, 12]. Notably, HPLC analysis has confirmed the presence of pharmacologically bioactive constituents including kaempferol, quercetin, amentoflavone, hesperidin, and protocatechuic acid in the leaves of the Cnidoscolus plant [15, 17]. These compounds are well-regarded for their anti-inflammatory, neuroprotective, cardioprotective, and antioxidant activities [17, 18, 20]. However, more research is required before concluding the specific contribution of these phytochemicals to the neuroprotective benefits of *CA* in rats following IR injury.

Conclusion: The results of this study revealed that post-treatment with *Cnidoscolus aconitifolius* attenuates neurological and behavioral deficits induced by the BCCAO global ischemic stroke model. *Cnidoscolus aconitifolius* provides comprehensive neuroprotection through combined antioxidant, anti-inflammatory, anti-apoptotic, and lipid-stabilizing effects, suggesting its therapeutic potential for mitigating neurological complications and promoting functional recovery after an ischemic stroke.

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