Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta Morphologica et Anthropologica, 31 (1-2)
Sofia ● 2024

Phyllanthus Amarus Mitigates against Potassium Dichromate-Induced Locomotion Posture and Coordination Impairment in Male Wistar Rats

Iteire Kingsley Afoke[1], Gbayisomore Tolulope Judah[1], Adunola Omosewa Victory[1], Babatunde Ogunlade[2], Dadeni Adeola Blessing[1]*

[1]Department of Anatomy, University of Medical Sciences, Ondo, Ondo State, Nigeria
[2]Department of Anatomy, Federal University of Technology Akure, Ondo State, Nigeria

*Corresponding author email: aiteire@unimed.edu.ng

The neuroprotective property of Phyllanthus amarus (AP) leaf extract was investigated in Potassium dichromate (PDCh)-induced cerebellar function impairment. The rats were divided into seven groups, including a control group, groups receiving different doses of AP, PDCh, and the drug donepezil (Dpz). Various tests assessed exploratory function, muscle strength, coordination, and spatial learning. The rats were examined, and their cerebellar tissues were analyzed. The PDCh group showed a significant decrease in body, brain, and cerebellar weight compared to the control, along with reduced grip strength, impaired locomotion, and increased freezing time in the open-field test. PDCh also affected T-maze and Morris water maze performance. Immunohistochemistry indicated cerebellar degeneration and altered expression of neuronal nuclear protein (NeuN) and Tissue Necrosis Factor TNF-α. GABA levels increased while Glutamate decreased, and Glutathione and Glutathione peroxidase were reduced in the PDCh group. Phyllanthus amarus treatment effectively countered these changes, offering protection against cerebellar degeneration and motor function impairment induced by PDCh toxicity.

Key words: Phyllantus amarus, neuroinflammation, neurotoxicity, motor function, cerebellum

Introduction

The cerebellum is responsible for the coordination of muscle movements, posture maintenance, and balance. For this reason, severe damage to the brain involving the cerebellum could impair its functions and result in ataxia. Cerebellar ataxia is a

progressive neurodegenerative disorder characterized by atrophy or degeneration of the cerebellum, leading to motor dysfunction, balance problems, and limb and gait ataxia [37]. Factors contributing to the pathophysiology of neurodegeneration of the cerebellum can be genetic, environmental such as (drugs, chemicals, metals, etc.), underlying chronic diseases, and multifactorial. Potassium dichromate (PDCh) is a heavy metal and widely recognized environmental pollutant linked to carcinogenic, teratogenic, and mutagenic effects [43]. It is mainly used as a precursor to potassium chromium alum in construction, photography, and printing. Like other hexavalent chromium compounds, PDCh is a strong oxidizer, acutely and chronically harmful to health. Widespread neurodegeneration has been observed in multiple studies involving the use of PDCh across multiple species, with significant toxicity to Purkinje cells of the cerebellum [9, 12]. This chemical compound generates reactive oxygen species (ROS), causing injury to the cellular proteins, lipids, and DNA and triggering a cascade of degenerating events leading to neuronal injury and neuroinflammation [38].

Moreover, neurodegeneration and neuroinflammation are intrinsically linked with associated inflammatory processes such as Alzheimer's disease (AD) and Parkinson's disease (PD). These two diseases of global importance, mainly to the elderly population, are estimated to have a death rate that has increased in both males and females within the last two to three years of the post-pandemic era [20]. Likewise, there is an overall ataxia prevalence rate of 26/100,000 in children, a prevalence rate of dominant hereditary cerebellar ataxia of 2.7/100,000, and a prevalence rate of recessive hereditary cerebellar ataxia of 3.3/100,000 [22]. Therefore, neurodegenerative diseases significantly impact the economy, and the risk of being affected increases with age, thus creating the need to improve our therapeutic approach towards them and adopt new ways to treat and prevent these neurological disorders. Besides, the use of plant materials as sources of medical compounds is rapidly emerging, and they have continued to play a dominant role in maintaining human health since antiquity.

Over 50% of all modern chemical drugs are of natural plant product origin and are essential in drug development programs of the pharmaceutical industry [40]. Therefore, intense research on the explorative use of orthodox medicine to treat neurodegenerative disorders (ND) is justified. Phyllanthus *amarus* (AP) is a widespread tropical plant known as the gale of the wind, stonebreaker, or seed-under-leaf. A link exists between its fruits as antioxidants, memory-enhancing, anticholinesterase, astringent, hepatoprotective, cytotoxic, and antimicrobial activity [28]. The phytochemical analysis of the plant extract revealed that it contains a high amount of saponins, tannins, flavonoids, and alkaloids [10]. Even though the neuroprotective nature of AP against oxidative stress has been identified in recent works, the specific dose range for its neuroprotective effect against neurotoxic agents is poorly understood, and its possible effect on potassium dichromate-induced neuronal cerebellar damage, about NeuN and TNFα expression, is yet to be documented. Therefore, this study will provide novel information on the neuroprotective effect of Phyllanthus *amarus* in PDCh-induced neurotoxicity in the cerebellum of male Wistar rats. Additionally, this study will further assess the neurobehavioral activity, oxidative stress level, GABAergic and Glutaminergic pathways, and immunohistochemically and histologically elaborate on new findings on the neuroprotective role of AP in the cerebellar impairment of rats treated with PDCh.

Materials and Methods

Aqueous extraction of Phyllanthus amarus leaves

Firstly, the fresh leaves were collected in bulk from a swamp around the Federal Housing Estate, Igba, Ondo City, Ondo State, Nigeria, and then immediately taken for identification by a plant Biologist at the Department of Plant Biology, Adeyemi Federal College of Education, with a Batch No: ACE/BIO/22/010. Plant authentication was carried out by a Herbarium Curator at the University of Medical Sciences, in the Department of Plant Biology and Biotechnology, with a herbarium Voucher label UNIMED/P.B.T.H/ 013, to preserve the voucher specimen for future reference. The harvested fresh leaves were then shade-dried for seven days with irregular sun drying for better grinding. The dried leaves were then ground into a coarse powder using a grinding machine and kept in air-tight conditions for extraction. The dried material (600 g) was macerated in 6 liters of distilled water for 48 hours at four °C in a refrigerator. The bottle's contents were sealed, kept at room temperature, and allowed to stand for seven days with irregular shaking. The extract was sieved, and the juice was filtered through Whatman (No. 1) filter paper. The filtrate was placed in a stainless-steel tray and concentrated in an air-circulating oven at 42°C until dry. The resulting extract (13 g) was placed into small glass dishes and stored at 28°C in an incubator for further studies. The different doses of 200, 300, and 400 mg/kg were reconstituted and stored in a stop-bottle for later administration via oral route to experimental animals [16].

Chemicals and reagents

Normal saline (100 ml) manufactured by Biomedical Limited, Nigeria, was purchased from Uche Care Pharmaceuticals Ondo City, Nigeria. Kermel Potassium dichromate (500g; molecular weight 294.18; UN number 3288) manufactured by Tianjin Kermel Chemical Reagent Co., LTD, China, was obtained from Pyrex Scientific Company, Benin Edo State, Nigeria. Donepezil hydrochloride 5mg tablets manufactured by Mutual Pharmaceutical CO., Inc., Philadelphia, USA, purchased from Uche Care Pharmacy LTD, Ondo City, Nigeria.

Experimental animals

The experiments were conducted at the Department of Anatomy, University of Medical Sciences (UNIMED), Ondo State, Nigeria. Animals were obtained from the Animal house's breeding colony of UNIMED and fed with standard rat chow (produced by Bendel livestock feed, Ibadan, Nigeria) and water throughout the study. Approval was granted by the Research Ethics Committee of the University of Medical Sciences, Ondo, Nigeria, with approval number NHREC/TR/UNIMED-HREC-Ondo St/22/06/21.

The animals received human care following the principle of human care and the use of laboratory animals. The rats were weighed weekly before the commencement and throughout the experiment using a Sunrise V-802 30Kg 1gm weighing scale manufactured by Sunrise Technology, USA, calibrated in grams, and recorded to the nearest whole number. Following acclimatization, seventy (70) young adult Wistar rats weighing between 100 g and 120 g were divided into seven groups of ten animals each. Experimental design and treatment protocol were shown on **Table 1**.

Table 1. Research design and treatment protocol.

S/N	Groups	Administration	Mode of Administration
A	Control	0.5ml of normal saline	Per Orally
B	Phyllanthus *amarus* only (AP)only	300mg/kg/day	Per Orally
C	Potassium Dichromate (PDCh)	17mg/kg/day	Per Orally
D	Donepezil only (Dpz)	5mg/kg/day	Per Orally
E	High Phyllanthus *amarus* + Potassium Dichromate	400mg/kg/day and 17mg/kg/day	Per Orally
F	Low Phyllanthus *amarus* + Potassium Dichromate	200mg/kg/day and 17mg/kg/day	Per Orally
G	Donepezil + Potassium Dichromate.	5mg/kg/day and 17mg/kg/day	Per Orally

Motor function tests: Neurobehavioral tests assessed how PDCh administration affects locomotor and exploratory activity.

Wire-hanging test: Commencing on the third day of the administration protocol, a wire-hanging test was conducted on rats to assess muscle strength following drug exposure. This wire suspension task gauges muscle strength and prehensile reflex, which is the animal's ability to grasp a tightly stretched horizontal wire with its forepaws and maintain suspension on the wire. The suspension time, which denotes the duration before the rat is released from the wire, was meticulously recorded. This parameter serves to identify neuromuscular abnormalities related to motor strength. A training session was conducted on the second day of the experiment to acquaint the rats with the task. Subsequently, the actual test started on the third day, as well as on days 7, 14, and 28. The cut-off time for the test was set at 180 seconds, indicating that rats were observed for a maximum of 180 seconds to remain suspended on the wire. If a rat dropped from the wire within this timeframe, the duration until the drop was noted [18].

Open field test (OFT): The OFT is a suitable measure of locomotion and anxiety in experimental animals (Millan, 2003). The OFT apparatus utilized in this study was a square wooden arena (72 cm × 72 cm × 20 cm) with lines on its floor dividing it into 18cm by 18 cm square [24]. The open field apparatus was cleaned with alcohol between each rat to avoid irritability due to odor. The rats were conveyed to the test room in their cages and tested for 5 minutes. Critical parameters of locomotor and exploratory activity, such as immobility (freezing time), rearing, ambulation, and grooming, were judiciously observed, and time spent executing each behavior was recorded.

T-maze spontaneous alternation test: T-maze spontaneous alternation is a behavioral test for measuring exploratory behavior in animals, especially in rodent models for CNS disorders. The test is based on the willingness of rodents to explore a

new environment, i.e., they prefer to visit a new arm of the maze rather than a familiar arm. Subjects were first placed in the start-arm of the T-maze. Upon leaving the start arm, subjects choose between entering the left or the right goal arm. With repeated trials, the animals should show less tendency to enter a previously visited arm. The percentage of alternation (number of turns in each goal arm) and total trial duration were recorded with a stopwatch. This test quantifies cognitive deficits in rats' transgenic strains and evaluates novel chemical entities for their effects on cognition [5].

Morris water maze: This test was carried out to assess the spatial learning and memory of the rats. A pool of water measuring about 100 cm in diameter and 30 cm in depth was used. An escape platform about an inch deep from the water's surface was placed outside one of the quadrants, which was a visual cue. The animals were trained 24 hours before the actual test. During the training, each rat was placed in the other three quadrants for a maximum of 60 seconds to find the escape platform at intervals of 15 minutes between quadrants until the escape latency period was reduced to less than 15 seconds. The time to find the escape platform was recorded as the escape latency period. The parameter measured in the Morris water maze is "Escape Latency" [8].

Determination of total brain and cerebellar weights

Morphometric analysis in this study involved the measurement of the rats' total brain weight (TBW) and cerebellar weight across experimental groups before brain dissection. The brain weight was measured using a sensitive weighing scale (HT-120 Electronic balance manufactured by AND Company Ltd, USA, 2014), and brain weights were recorded. Afterward, the brain was dissected into specific regions; the cerebellum was weighed using a sensitive weighing scale HT-120 Electronic balance manufactured by AND Company Ltd, USA, 2014). The mean ± SEM was calculated using the GraphPad Prism 8.0.1.244 x86 software.

Histological processing/biochemical analysis

After the conduct of the behavioral assessment, the rats were subjected to cervical dislocation; after the conduction of morphometric analysis, the brain tissues were then excised and dissected. Coronal sections of the cerebellum were obtained stereotaxically. For each group, three rat brain samples were chosen for the immunohistochemistry process, targeting NeuN and TNFα expression. Additionally, three brain samples per group were used for biochemical analyses, and another three brain samples per group were allocated for histochemistry to assess neurotransmitter levels. The cerebellum structures dissected from the brain samples were then embedded in an EDTA sampling bottle containing 10% neutral buffered formalin to preserve it. Subsequently, this part of the brain was processed to obtain a Paraffin wax-embedded block for H & E demonstration [15]. The homogenate of this tissue was obtained and then centrifuged at 12,000 rpm to obtain the supernatant containing tissue lysates. The supernatants were obtained and stored at very low temperatures. Glutathione peroxidase GPx activities in the tissue lysates were assayed using appropriate enzyme lysate immunosorbent assay kits.

Immunohistochemistry

NeuN: Immunohistochemistry analysis was performed to selectively identify antigens (proteins) on cells in a tissue section by exploiting the principle of antibody binding

specifically to antigens on biological tissues. The cerebellum was fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin wax. Sections of 3 μm thicknesses were cut onto a charged-slides, deparaffinized, and rehydrated. Heat-mediated antigen retrieval was performed using a citrate-based antigen retrieval solution (pH 6.0) for 30 minutes. Endogenous peroxidase blocking (10 min) was performed before protein blocking (10 min). Immediately after, it was followed by incubation with the primary antibody's anti-NeuN antibody at 1:1000 for three hours at room temperature. Sections were then incubated in HRP (horse radish-peroxidase) micro-Polymer Goat Antirabbit HRP (Vector USA) for 30 minutes. The reaction was developed with DAB chromogen (Vector, USA). Sections were then rinsed in water, counterstained with Hematoxylin, dehydrated, cleared, and mounted with Dibutyl Phthalate Xylene (DPX). Thermofischer Scientific Inc. manufactured the enzyme immunoassay kit used (United Kingdom).

*TNF-alpha (α)***:** Levels of TNF-α were measured using a Tumor necrosis factor-alpha Assay kit (chemiluminescent assay) through the IMMULITE® 1000 system according to the manufacturer's instructions. The detection limit of the kits was obtained from the manufacturers as TNF-α (detection limit 1.7_1000pg/mL).

Assessment of neurotransmitters

Glutamate: Brain tissue was deproteinized according to a modification of the method of [2]. Each brain was rapidly weighed, and ground to a powder in a stainless-steel tissue grinder cooled in liquid nitrogen. Five hundred microliters of 2N HCO4 were frozen and ground in a separate stainless-steel tissue, grinder cooled in liquid nitrogen. The two frozen powders were combined in a plastic centrifuge tube and allowed to warm to 4°C. One and a half to two milliliters of Ice-cold deionized H_20 was added. Ten minutes later, the content was homogenized for 60 sec with a motorized. Teflon tissue grinder. The homogenate was centrifuged at 3000g for 15min at 0°C. The supernatant was removed. Neutralized with 2N KHC03 and centrifuged again under the same conditions. Half of the final supernatant was stored at -20°C for glutamate assays. The remaining supernatant for the α-KGA assays was stored on dry ice (-78.5°C). Glutamate concentrations were determined by modifying the method of [2]. The principle of this enzymatic spectrophotometric assay is that the reduction of 3acetylpyridine adenine dinucleotide (APAD) to APADH is proportional to the amount of glutamate present. The amount of APADH formed during the assay was determined by measuring the increase in absorbance at 363.

Gamma-aminobutyric acid (GABA): This assay employs the competitive inhibition enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with Gamma-Aminobutyric Acid (GABA) protein. Standards or samples are added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to Gamma-Aminobutyric Acid (GABA). Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After the TMB substrate solution is added. Adding sulphuric acid solution terminates the enzyme-substrate reaction, and the color range is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of Gamma-Aminobutyric Acid (GABA) in the samples is then determined by comparing the OD of the samples to the standard.

Determination of glutathione peroxidase (GPx) and GSH activity

GPx activity was determined according to the method of [31]. Glutathione peroxidase activity was observed by plotting a standard curve, and the concentration of the remaining GSH was extrapolated from the curve.

$$\text{GSH consumed} = 245.84 - \text{GSH remaining}$$

$$\text{Glutathione peroxidase activity} = \frac{\text{GSH consumed}}{\text{mg protein}}$$

Photomicrography and data analysis

Photomicrographs of slides obtained from histological and immunohistochemical processing of tissues were captured using a binocular microscope connected to a 5.0-megapixel camera. Data obtained from enzyme analysis was subjected to statistical analysis using the graph Pad Prism software (version 5). The results generated from the cognitive studies, immunoquantification of NeuN and TNF-α, and results of GPx, GSH Glutamate, and GABA concentrations were plotted as bar graphs with error bars (mean and standard error of the mean). One–way ANOVA followed by the Tukey test was used to determine any significant difference among the groups with a confidence limit of 95%. Data represent mean value ± SEM.

Results

The rats in the control group maintained a relatively average weight of 120-152 g throughout the experiment, similar to the positive control groups (AP and Dpz only). Rats treated with AP for 28 days showed no abnormal behavioral changes while handling and home-cage movement. After the dissection of the animals, the brain structure was observed to identify if the AP had caused any morphological effect on it. The animals from AP groups showed no edema or inflammatory changes in the brain structure.

Body weight and brain morphometric comparison (brain and cerebellar weight) across the groups were shown on **Fig. 1(a-c)**. Control and AP showed no significant difference in body weights. However, the body weight of PDCh group was significantly reduced compared to the control. Additionally, the Dpz group, AP (400 mg/kg) + PDCh and Dpz +PDCh showed a significant increase ($p < 0.001$) compared to the control group. In contrast, the AP (200 mg/kg) + PDCh was significantly reduced ($p \leq 0.001$) compared to the Dpz + PDCh. Noteworthy, is that the AP-treated group maintained an average weight throughout the study, and AP protected against weight loss in the PDCh-treated rats (**Fig. 1a**). Brain morphometric analyses also revealed a significant decrease ($p < 0.01$) in the brain weight of the PDCh-only-treated group compared to the Control group. Additionally, significant increments were observed at $p < 0.01$ and $p < 0.001$ when comparing the AP (400 mg/kg) + PDCh and the AP (200 mg/kg) + PDCh as well as Dpz groups to the PDCh group, respectively (**Fig. 1b**). Also, a significant decrease ($p < 0.01$) in cerebellar weight was observed in the PDCh-only group compared to the control group and Dpz (**Fig. 1c**). However, AP protective

treatment group (200mg/kg), comparably with Dpz, improved markedly in the brain and cerebellar weight loss caused by PDCh.

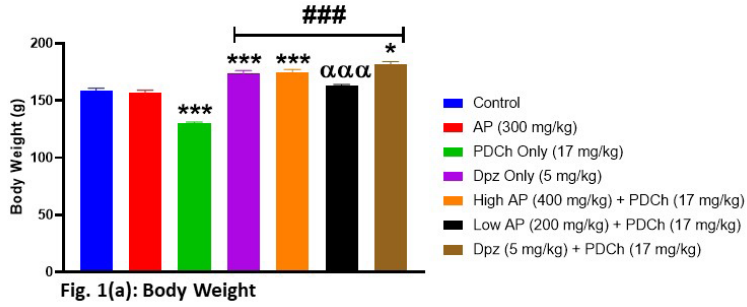

Fig. 1(a): Body Weight

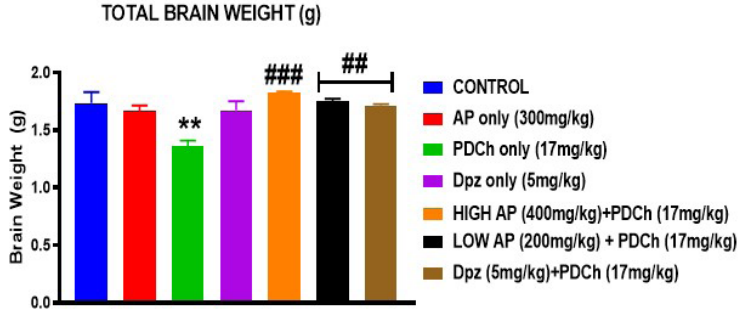

Fig. 1(b): Total Brain Weight

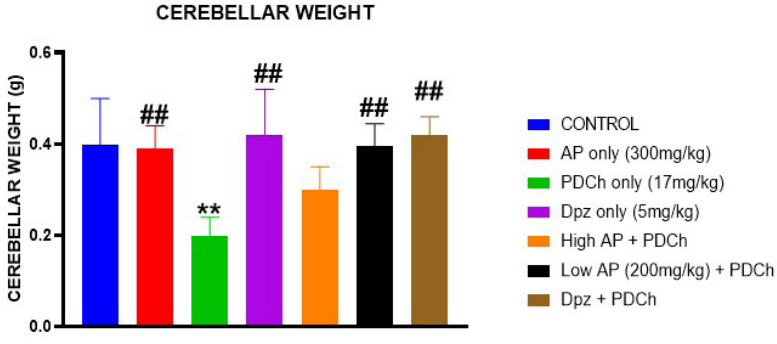

Fig. 1(c): Cerebellar Weight

Fig. 1. Changes in total brain and cerebellar weight. (a) body weight; (b) brain weight; (c) cerebellum weight.* denotes significant difference at $p<0.05$ when compared to control group, ** denotes significant difference at $p<0.01$ and *** at $p<0.001$ when compared to control group; ## denotes significant difference at $p<0.01$ and ### at $p<0.001$ when compared to PDCh only group; ααα denotes significant difference at $p<0.001$ when compared to Dpz + PDCh group.

Time spent hanging on a wire in the Wire hanging task was shown on **Figure 2**. There is no significant difference in time spent hanging on the wire ($p > 0.05$) between the control and PDCh group on the third day of administration, as well as other experimental groups. On day 7, a significant increase ($p < 0.01$) in the Wire hanging period was seen in the AP group compared to the Control group and the PDCh group. On day 14, no significant decrease ($p > 0.05$) in the Wire hanging period was seen in the PDCh group compared to the Control group. On day 28, a significant decrease ($p < 0.05$) in the Wire hanging period was seen in the PDCh group compared to the Control group. Nonetheless, a notable but statistically insignificant reduction was observed in the PDCh group when compared to the other experimental groups. Notably, AP treatment significantly improved the time spent hanging on the wire in the rats treated with PDCh, showing similar improvements as observed with Dpz across the experimental days.

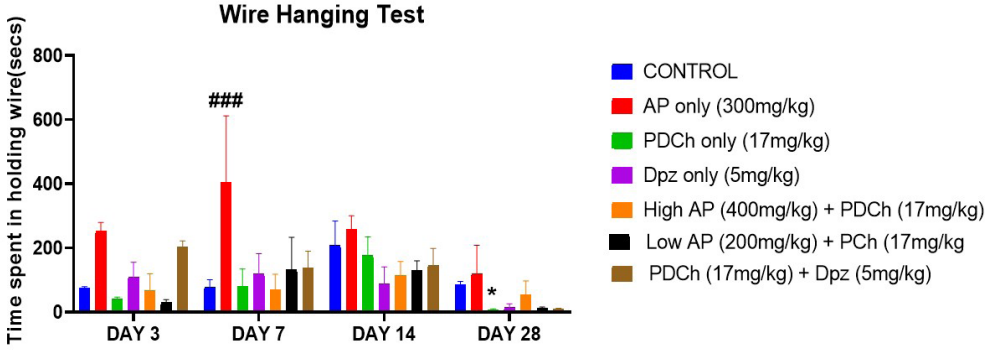

Fig 2. Wire Hanging test across administration days. * denotes significant difference at $p<0.05$ compared to control group; ### denotes significant difference at $p<0.001$ when compared to PDCh group.

The activities of experimental rats in the Open field task were shown on **Figure 3**. No significant variance ($p > 0.05$) was seen between the Control group and other experimental groups on day seven in the number of lines crossing as an exhibition of locomotive behavior **(Fig. 3a)**. On day 14, no significant difference ($p > 0.05$) in line crossings was observed in the PDCh group compared to the control group. On day 28, a significant reduction ($p < 0.01$) in line crossings was seen in the PDCh compared to Control and AP. A significant and noteworthy finding was the substantial reduction ($p < 0.001$) in the number of line crossings in the PDCh group in comparison to the High AP (400 mg/kg) + PDCh group. Also, in the rearing behavior, on days 14 and 28, a significant reduction ($p < 0.05$ and $p <0.01$, respectively) was seen in the PDCh group compared to the control group and the High AP (400 mg/kg) + PDCh (**Fig. 3b**). Also, there were significant decrease ($p < 0.05$) in number of rearings of PDCh group compared to the AP and Dpz groups. However, no significant difference was seen across the experimental groups compared to the Control in the Time spent in the centre square (**Fig. 3c**). In the frequency of entries into the center square, an obvious distinction was seen on day 28 in the PDCh group compared to other experimental groups, with a significant reduction ($p < 0.01$) in the PDCh group compared to the

AP only group (**Fig. 3d**). In the fecal boli count, no significant difference was seen across the groups (**Fig. 3e**). However, relatively high Fecal boli on day 14 and 28 can be observed in the PDCh and Dpz + PDCh groups compared to the control, indicating the exhibition of fear or anxiety. The freezing time indicates the period of immobility (**Fig. 3f**). A significant increase ($p < 0.05$) was observed in the PDCh group compared to the control group on day 14. Similarly, on day 28, a markedly significant increase ($p < 0.001$) was observed in the PDCh group compared to the control group and other experimental groups, indicating a deteriorating effect on the motor function of the treated rats as the administration progresses. The administration of AP demonstrated a protective effect against the motor function impairments induced by PDCh treatment in the rats. No significant difference ($p > 0.05$) in freezing time seen between AP + PDCh treatment groups and the Dpz + PDCh standard group was observed.

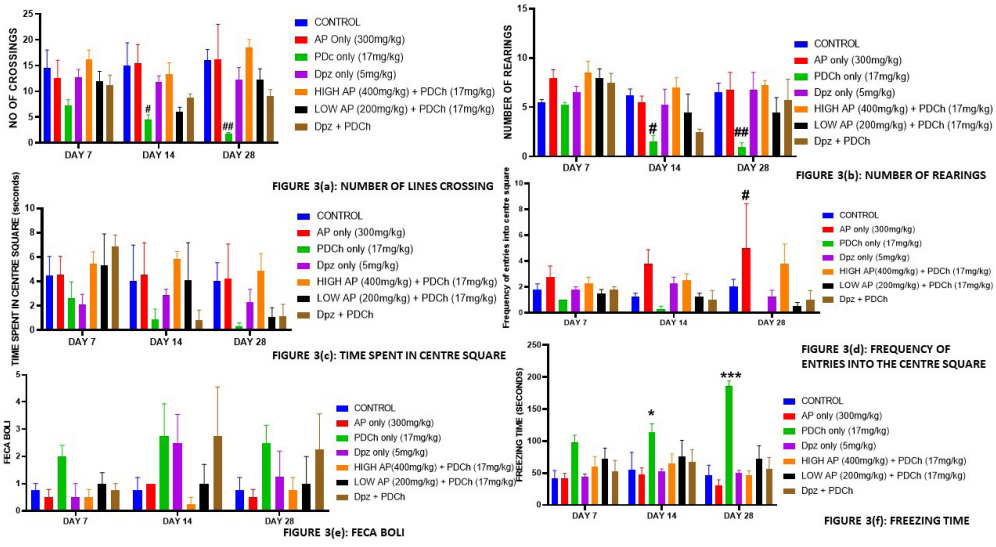

Fig. 3. The activities of experimental rats in the open field task. (a) number of lines crossing; (b) number of rearings; (c) time spent in center square; (d) frequency of entries into the center square; (e) fecal boli; 3(f) freezing time. * denotes a significant difference at $p<0.05$ and *** at $p<0.001$ when compared to control group; # denotes a significant difference at $p<0.05$ and ## at $p<0.01$ when compared to PDCh only group.

The frequency of entries in the T-maze task (a) and variations in Escape latency (EL) in the Morris water task (b) across treatment groups were shown on Figure 4. Significant difference ($p < 0.05$) was seen in the percentage frequency of arm entries on day seven between the PDCh group and the High AP + PDCh group**.** Although, no significant difference ($p > 0.05$) was seen across the experimental groups on days 7 and 14, a progressive reduction in the frequency of arm entries was noticed in the PDCh group on days 7and 14, with a significant reduction ($p < 0.05$) seen in PDCh compared to the control group on day 28 (**Fig.4a**)**.** However, no significant difference was seen between the Dpz + PDCh, High AP+ PDCh, and Low AP + PDCh groups compared to the control. This depicts the protective effect of AP against PDCh motor function

damage in rats seen on day 28 in both doses of AP. On day 7, a significant difference ($p < 0.05$) was seen in the Dpz + PDCh group when compared with the control group (**Fig. 4b**). Also, no significant differences were seen between the Dpz + PDCh group when compared with the High AP + PDCh group and the Low AP + PDCh group. On day 28, a significant difference ($p < 0.05$) was seen in the control group and AP group compared to the PDCh-only group, which reported the highest EL. Also, it's worth noting that a significant decrease ($p < 0.05$), reflecting a shorter escape latency, was observed in the 400 mg/kg AP group when compared to the Dpz + PDCh group. Interestingly, there was no significant difference between the 200 mg/kg AP and Dpz + PDCh treated groups, both of which maintained an average escape latency throughout the study.

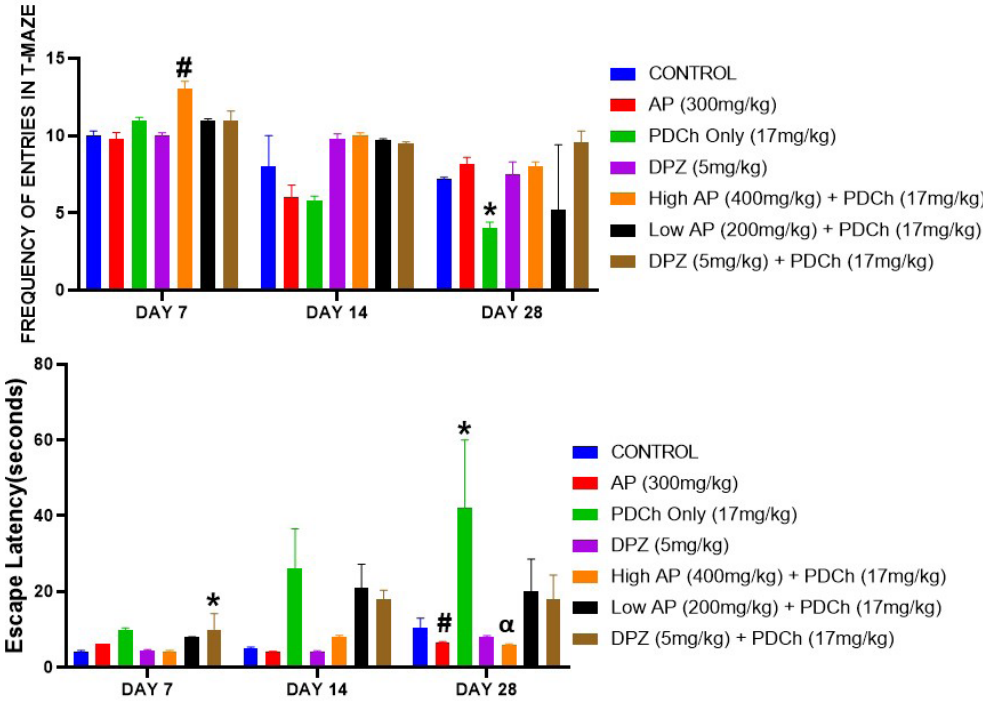

Fig. 4. (a). Frequency of arm entries in T maze; **(b).** Escape latency in Morris water maze

Fig. 4. Bar chart showing (a) Frequency of arm entries in T maze; (b) Escape latency in Morris water maze. *denotes significant difference at $p<0.05$ compared to the control group; # denotes significant difference at $p<0.05$ compared to PDCh only group; **α** denotes significant difference at $p<0.05$ when compared to Dpz + PDCh group.

Photomicrographs of the cerebellar histological architecture were presented on **Figure 5.** The cerebellar histology of the cerebellum shows three distinct layers: the granule layer with small, tightly packed rounded granular neuron; the Purkinje layer, which consist of a single layer of the large round nucleus and prominent nucleoli; the molecular layer with sparse cells. Control, AP, and Dpz groups show relatively intact cerebellar histology. Also, the High AP (400mg/kg) + PDCh and Dpz + PDCh

groups show typical cerebellar neuronal structures, depicting protection against PDCh cerebellar toxicity. The Low AP + PDCh shows normal neuronal structure but signs of cellular shrinking and scattered neurons. However, unlike other treatment groups, the PDCh group shows apparent degenerative features characterized by cellular shrinking, nuclear fragmentation, and neuronal degeneration.

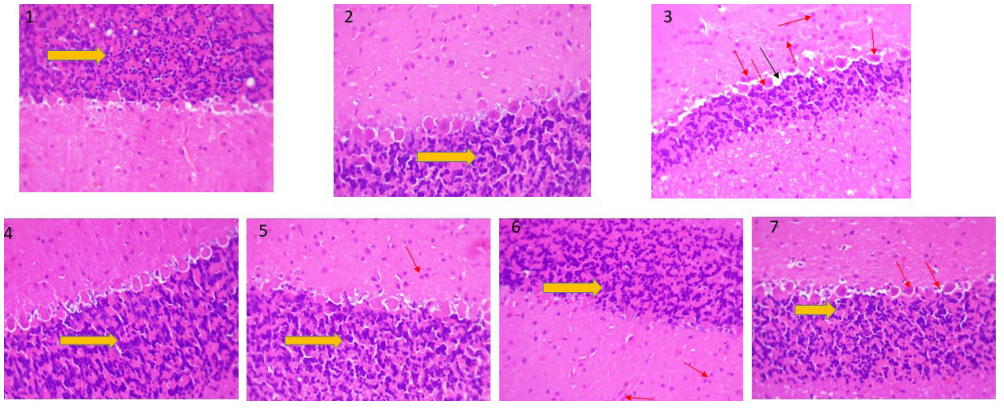

Fig. 5. Photomicrographs of Haematoxylin and Eosin staining of the cerebellum. Control (1), AP (2), PDCh (3), Dpz (4), High AP + PDCh (5), Low AP + PDCh (6), and Dpz + PDCh (7) groups (H and E, x400). Yellow arrows indicate a normal cerebellar neuronal structure. Black arrows indicate degenerating neurons at Purkinje layer with appearance of vacuolation. Red arrow denotes nuclear fragmentation.

Cerebellar immunohistochemical visualization of NeuN was presented on **Figure 6**. Chart shows the quantification of the percentage of NeuN expression in control and treated groups. The immunohistochemical localization of NeuN shows distinct expression in cerebellar neurons. PDCh-only groups show markedly reduced expression of NeuN immunoreactive neurons compared to the Control, AP, Dpz group, High AP PDCh, Low AP + PDCh, and Dpz + PDCh group. This result suggests that PDCh significantly reduced NeuN expression. In contrast, treatment with both Dpz, High and Low doses of AP significantly increased Neun expression compared to PDCh-only group.

Cerebellar immunohistochemical visualization of TNFα was presented on **Figure 7**. Chart shows the quantification of the percentage of TNFα immunoreactivity in control and treated groups. The percentage of TNFα immunoreactive cells in PDCh, Dpz, and Dpz + PDCh groups was significantly higher ($p < 0.001$) than in Control, AP, High AP + PDCh, and Low AP + PDCh groups. However, there is a significant reduction of TNFa expression ($p < 0.001$) in the AP group and AP + PDCh group compared to the PDCh group. Similarly, there was decreased expression ($p < 0.01$) in the PDCh + Low AP groups when compared to the PDCh group. Significantly, the expression of TNFα in both the 400 mg/kg and 200 mg/kg AP + PDCh groups showed a substantial reduction ($p < 0.001$) when compared to the Dpz + PDCh group. This result suggests that PDCh and Dpz or a combination of PDCh + Dpz may significantly increase TNFα expression. In contrast, treatment with high and Low doses of AP sufficiently reduced TNFα expression.

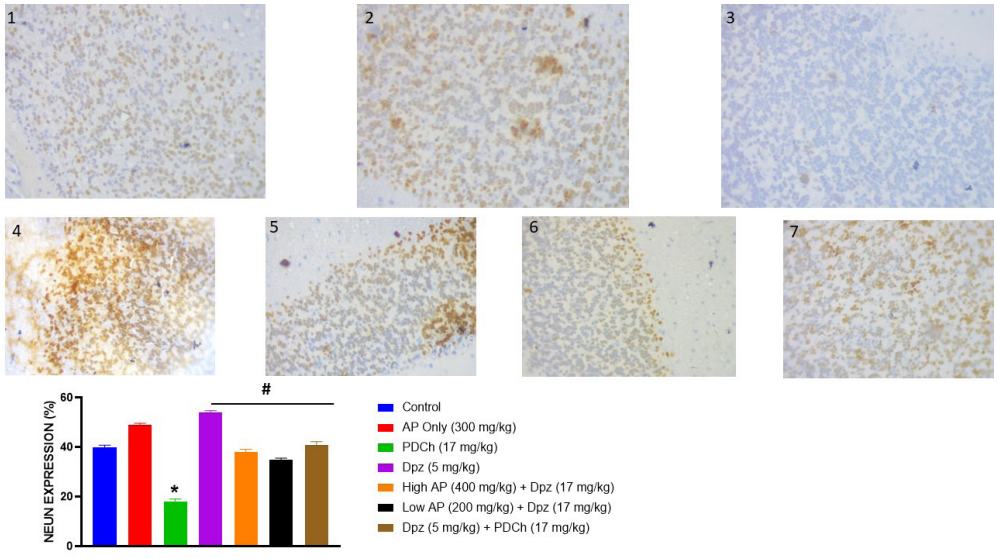

Fig. 6. Protein expression of NeuN in the cerebellum. (1-7) are photomicrographs as follow: Control (1), AP (2), PDCh (3), Dpz (4), High AP + PDCh (5), Low AP + PDCh (6), and Dpz + PDCh (7) groups (x400); Chart shows quantification of NeuN protein expression * denotes significant difference at $p<0.05$ when compared to the control group; # denotes significant difference at $p<0.05$ when compared to the PDCh only group.

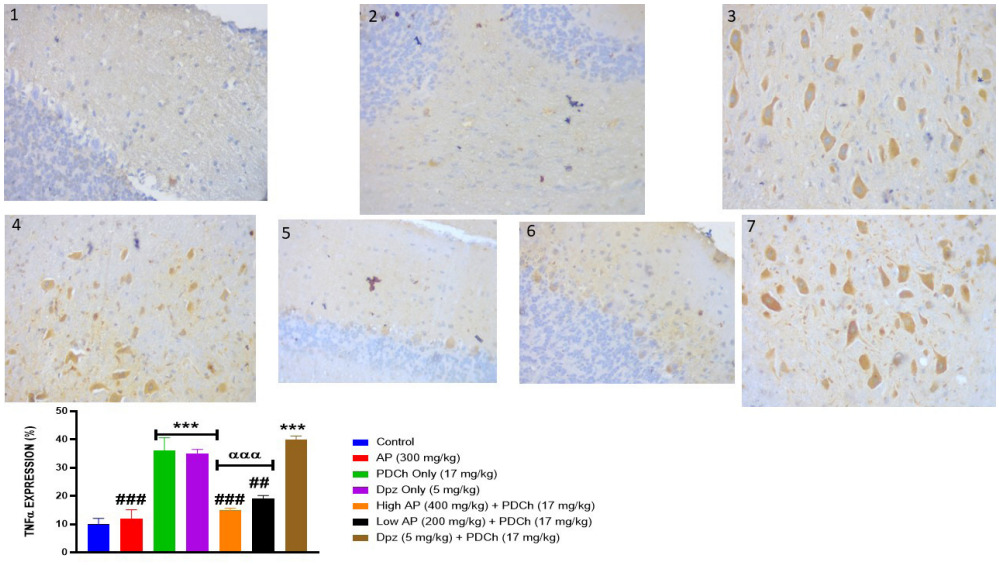

Fig. 7. Protein expression of TNF-ά in the cerebellum. (1-7) are photomicrographs as follow: Control (1), AP (2), PDCh (3), Dpz (4), High AP + PDCh (5), Low AP + PDCh (6), and Dpz + PDCh (7) groups (x400). Chart shows quantification TNF-ά expression in the cerebellum; *** denotes a significant difference at $p<0.001$ when compared to control; ## and ### denotes significant difference at $p<0.01$ and at $p<0.001$ when compared to PDCh only group; ααα denotes significant difference at $p<0.001$ when compared to Dpz+PDCh group.

Figure 8 demonstrated our data from measurements of Glutamine (a) and γ-aminobutyric (GABA) (b) levels across experimental groups. Significant difference ($p < 0.05$) in the Glutamate level in the control and AP groups was seen when compared to the PDCh group (**Fig. 8a**). Also, the result reveals no significant difference between High AP (400 mg/kg) + PDCh and Dpz + PDCh groups, but relatively reduced Glutamate level in the Low AP (200 mg/kg) + PDCh group compared to the control group, suggesting dose-dependent protection. Significant increase ($p < 0.001$) was found in GABA levels of the PDCh group compared to the control group and other experimental groups (**Fig. 8b**). However, no significant difference ($p > 0.05$) was seen between High AP + PDCh, Low AP +PDCh, and Dpz + PDCh.

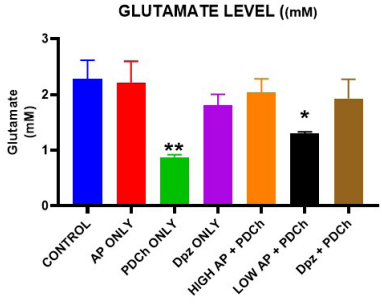

Fig. 8(a). Glutamate levels

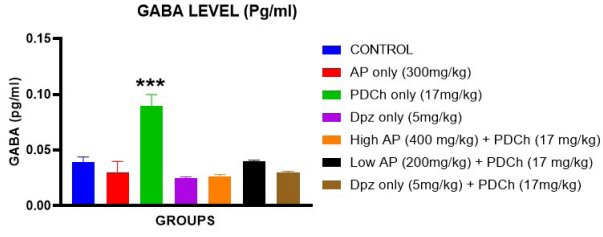

Fig. 8(b). GABA levels

Fig. 8. Quantification of glutamate (**a**) and GABA (**b**) levels across experimental groups. * denotes a significant difference at $p<0.05$, ** at $p<0.01$ and *** at $p<0.001$ when compared to the control group.

Figure 9 demonstrated concentration of Glutathione peroxidase (GPx) (a) and Reduced Glutathione level (GSH) (b) across experimental groups. No significant difference ($p > 0.05$) in the Glutathione peroxidase level was observed between the control and the PDCh groups (**Fig. 9a**). Also, the result reveals no significant difference between High AP (400 mg/kg), Low AP (200 mg/kg), and Dpz + PDCh group compared to the control group. Significant increase ($p < 0.001$) in the Reduced Glutathione level was observed in the control group and AP group when compared to the PDCh group. The result also reveals a significant increase ($p < 0.05$) between the Low AP + PDCh and control groups. The levels of Reduced Glutathione level across AP and Dpz groups are relatively average and similar to the Control group.

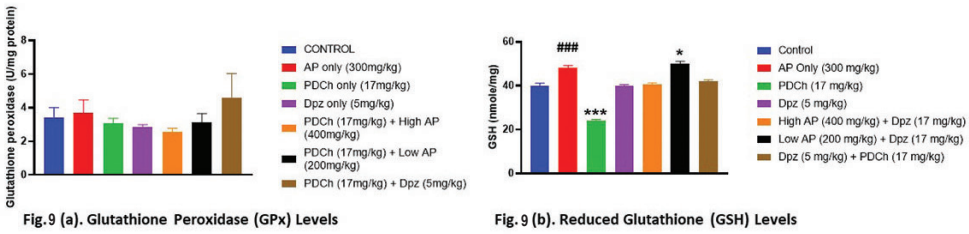

Fig.9 (a). Glutathione Peroxidase (GPx) Levels

Fig. 9 (b). Reduced Glutathione (GSH) Levels

Fig. 9. Quantification of Glutathione Peroxidase and Reduced Glutathione levels across experimental groups. * denotes significant difference at $p<0.05$ and *** at $p<0.001$ when compared to control group; ### denotes significant difference at $p<0.001$ when compared to PDCh only group

Discussion

In this study, the body weight was measured on the final day of administration to assess the differences in weight across experimental groups. Body weight is an indicator in examining motor balance and monitoring disease progression [6]. Moreover, reduction in body weight occurs frequently during advanced stages of spinocerebellar ataxia type 2(SCA2) [30]. In our study, a significant reduction in body weight was observed in the PDCh group when compared to both the control group and the other experimental groups. This suggests an early onset of ataxia, which notably impacted body weight. However, the AP-treated groups provided protection against changes in body weight in a dose-dependent manner, countering the deteriorating effect of PDCh on body weight.

Furthermore, total brain weight and cerebellum were weighed to assess brain morphological changes. The results showed a significant reduction in the total brain and cerebellar weights in the PDCh group compared to the control group. Reduction in brain weight is an apparent cause of disruption of the cerebellar hemispheres and dentate nuclei, which may result in limb ataxia, hypotonia, terminal and intention tremor, and dysarthria [3] — in addition, reduced cerebellar weight results from cerebellar degeneration, which leads to difficulty in balancing and coordination. Hence, this claim establishes that PDCh groups affected the total brain weight with edema changes seen morphologically and cerebellar weight reduction. Furthermore, in this study, AP and Dpz protected against cerebellar degeneration and brain toxicity caused by PDCh.

The wire-hanging test is widely used to measure rats' motor activity, coordination, and endurance [18]. The cerebellum, known as the "little brain, maintains balance and motor coordination. The wire hanging test was employed after a pre-determined treatment with PDCh and, subsequently, AP to ascertain its motor function. Findings showed marked reduced motor coordination and balance after PDCh exposure in rats. This reflects underlying progressive cerebellar dysfunction because the time of hanging to the suspension reduced as the administration of PDCh progressed. However, AP treatment improved the motor coordination and balance of the PDCh-induced rats, similar to the Dpz protective treatment group, as indicated by an increased time hanging on the wire. This observation was further supported by the open field test (OFT).

For instance, rearing and line-crossing behavior and activities in the centre-square have been reported to be potent indicators of locomotor and exploratory behaviors, respectively; immobility (freezing time) and fecal boli count are positively correlated to fear or anxiety [33]. Our findings on increased freezing time (immobility) agree with the findings of other studies, which demonstrated increased immobility following metal exposure in rats [25, 36].

Furthermore, impairment of locomotor and exploratory functions in the experimental rats is confirmed with the decreased crossing and rearing behaviors and fewer activities in the center square of the OFT apparatus. This finding also resonates with that of [41], who observed a positive correlation between line crossings and rearing. The findings revealing the motor function-enhancing effect of AP are supported by claims that plant extracts can mitigate metal toxicity in experimental rats [26, 32]. Besides, existing studies reported that aqueous Phyllanthus *amarus* extract (100 and 200 mg/kg) and methanolic extract of Phyllanthus *nuriri* enhanced neuromuscular coordination and balancing [1]. Nonetheless, this study explores the doses 200 and 400mg/kg dose, and new parameters were used in the OFT test, including Fecal boli count, to examine coordination. This study's findings show that PA attenuated PDCh-induced neurotoxicity; we suspect the plant's antioxidant properties as an antioxidant and acting as a neuroprotective agent in a dose-dependent manner.

The T-maze results show that PDCh affected the locomotive activity of the treated rats, exhibited by the significant reduction in the frequency of arm entries on between days 14 and 28. This correlates with the findings of [14], who reported that Hexavalent chromium caused hypo locomotive behavior in Hexavalent chromium-treated mice. About other experimental groups, our results show that AP possesses great locomotive-enhancing activity comparable with Dpz, which also demonstrated a cognitive protecting effect, as seen in the peak of arm entry frequency on day 7 in the AP + PDCh group. Similarly, in the Morris water maze task, an elevated level of escape latency was observed in the PDCh-treated rats, indicating cognitive impairment in decision-making and spatial awareness. PDCh rats swam for extended periods and traveled farther before locating the platform. These impairments were not improved when treated with Low AP (200 mg/kg) + PDCh group; however, when the dose was increased to 400 mg/kg and 300 mg/kg), there was enhanced cognitive function to exit the maze in PDCh-treated rats, which exhibited reduced levels of escape latency comparably better than Dpz (5mg/kg) treatment. These findings correlate with [27], which reports the neuroactive effects of Phyllanthus *amarus* (400 and 200 mg/kg) assessed under the Morris water maze task. However, this present study also supports the claim and asserts the cognitive-enhancing effect of 300 mg/kg Phyllanthus amarus aqueous extract in the Morris water maze task. Therefore, AP sufficiently protected against cognitive dysfunction induced by PDCh in rats.

The study also revealed that H & E stained cerebellar sections disclosed intact Purkinje neurons for both the control and the AP Only group. However, the PDCh-only group showed marked neurodegenerative changes in the Purkinje and granular layers. Additionally, signs of cell death and a faint nuclear outline of the Purkinje cell indicated early signs of neuronal nuclei destruction. These features observed by staining with H & E are consistent with reports of [9], and further correlated with the findings of our NeuN immunohistochemistry. Neuronal nuclear protein (NeuN) is a well-known marker found exclusively in post-mitotic neurons. It is found in the nucleus

of mature neurons and readily visualized with immunostaining. Thus, the current study used NeuN expression to demonstrate the effect of PDCh on the number or health of mature neurons in the cerebellar neuronal population. Our findings revealed that NeuN expression was significantly down-regulated in PDCh-treated rats, indicating occurring degenerative changes in the cerebellum or, neuronal development or maturation being disrupted, and neurons not reaching their mature state. Surprisingly, AP and Dpz maintained the regular expression of this protein relative to the control group.

Furthermore, it has been proven that chronic inflammation in specific brain areas is common in patients with cerebellar dysfunction. This led to the investigation of some anti-inflammatory drugs, such as Indomethacin, to halt inflammatory diseases [29]. Likewise, in our study, TNF-α expression was assessed, and AP was shown to exhibit a protective effect against an inflammatory factor (TNF-α) in the cerebellum, which is known to damage neurons to a certain extent. TNF-α in the Dpz + PDCh and PDCh groups were highly expressed compared with the control group, which suggested that the cognitive decline might have been caused by PDCh triggering microglial activation. However, the highly expressed TNF-α seen in the Donepezil-treated group is not correlated with past findings. Although Dpz has not been observed to augment the mRNA expression of anti-inflammatory phenotypes in primary microglial cells as a neuroprotective drug, it is expected to suppress the expression of TNF-α [13, 39]. This opens a research knowledge gap on the possible astrocytic mechanism involved in the anti-inflammatory effects of Dpz.

Glutamate is the most abundant excitatory neurotransmitter in the brain, and GABA is an inhibitory neurotransmitter. Glutamate/GABA concentration imbalance leads to progressive cognitive disorders [11]. Thus, Glutaminergic/GABAergic system balance is needed to attain optimal cognitive function and to keep the brain functioning correctly. This study observed that the glutamate concentration was markedly reduced in the PDCh-treated group compared to the control group. There is a link to cognitive deficits being associated with glutaminergic hypofunction [21].

Moreover, Ciacci et al. [4] reported that hexavalent chromium induces neurotoxicity by inhibiting Acetylcholine through alteration in its synthesis. Hence, it is imperative to note that PDCh caused glutaminergic hypofunction in this study and confirms the claim that PDCh causes inhibition of glutamine neurotransmitters. Surprisingly, AP protective treatment groups [300mg/kg and 400mg/kg] maintained balanced glutamate levels similar to the control. Also, this study demonstrated a reduction in GABA levels in the PDCh-only treated group compared to the control. Behavioral analysis revealed that reduced GABA+ levels were associated with decreased cognitive function. This proves that dysfunction of the GABAergic inhibitory circuits in the cerebellum contributes to changes in cerebellum neuronal excitability and cognitive impairment, leading to the inability to execute normal adaptive behavioral responses. Therefore, treatment with AP protected maintained balance in Glutamate/GABA signaling, and PDCh distorted Glutamate/GABA signaling, resulting in decreased motor activity in the OFT.

Glutathione peroxidase is an antioxidant-dependent enzyme important in the antioxidant defense mechanism. Oxidative stress and antioxidant levels are markers in inflammatory reactions. Glutathione peroxidase has been extensively examined in the cerebellum of PD, and there is wide variability in its reported levels and activity. This enzyme has been localized exclusively in the glial cells and down-regulated in astrocytes and neurons due to oxidative stress [35]. However, its levels and activity in

our study are generally not markedly elevated. There was no significant variability in Glutathione peroxidase across the experimental groups.

Conversely, Glutathione peroxidase level was similar across all the other AP and Dpz-treated rats and in the control group, suggesting oxidative stress support. Similar results were obtained on administering Phyllanthus *amarus* extract against neurotoxic agents [19, 23], although the studies only considered AChE inhibitors on the oxidative pathways.

GSH plays a vital role in neuronal defense against damage caused by oxidative agents like ROS and RNS [42]. Its depletion is implicated in cerebellar dysfunction, cerebral palsy, spinocerebral degradation, and ataxia [7]. Moreover, decreased GSH levels elevate cellular vulnerability towards oxidative stress and accumulating oxidative stressors in the substantia nigra of PD patients, brains of epileptic humans, and genetically epileptic transgenic mice [17]. Consequently, this antioxidant is vital in the onset and progression of neurological disorders and neurodegenerative diseases and serves as a biomarker for diagnostic screening. This present study showed a markedly significant reduction in the GSH level of the PDCh group, indicating oxidative stress and reduced antioxidant levels. However, GSH was significantly increased in the Low AP + PDCh group compared to the control group. This finding implies that at low doses of AP, GSH is neuroprotective. However, at high (Mm) concentrations, GSH may affect the redox state of glutamate receptors via its free thiol group [34]. This result corroborates earlier glutamate findings in which Low AP (200 mg/kg) + PDCh was distorted with substantially reduced Glutamate levels compared to other AP and Dpz treated groups. However, the Glutathione peroxidase level was maintained across all other AP and Dpz-treated rats in the control group, suggesting an anti-oxidative maintaining property of AP.

Conclusion

In conclusion, this study provides behavioral, histological, immunohistochemical, and neurotransmitter evidence that the aqueous extract of Phyllanthus *amarus* leaves extract is anti-inflammatory and significantly reduces the neurotoxic effect of potassium dichromate by mitigating locomotion, posture, and coordination impairment.

Acknowledgment: The authors appreciate The NeuroLab, Federal University of Technology Akure, for technical support.

References

1. **Ambali, S. F., A. O. Makinde, M. Shittu, S. A. Adeniyi, F. O. Mowuogwu.** Mitigating effects of *Phyllanthus niruri* on sensorimotor and cognitive impairments induced by subacute chlorpyrifos exposure in Wistar rats. – *Am. J. Med. Med. Sci.*, **2**, 2012, 50-58.
2. **Bernt, E., H.-U. Bergmeyer.** l-Glutamate: Determination with glutamic dehydrogenase. In: *Methods of enzymatic analysis* (Eds. H. U. Bergmeyer), Academic Press Inc, 1965, 384-397.
3. **Bodranghien, F., A. Bastian, C. Casali, M. Hallett, E. D. Louis, M. Manto, P. Mariën, D. A. Nowak, J. D. Schmahmann, M. Serrao, K. M. Steiner, M. Strupp, C. Tilikete, D. Timmann, K. van Dun.** Consensus paper: Revisiting the symptoms and signs of cerebellar syndrome. – *Cerebellum*, **15(3)**, 2016, 369-391.

4. **Ciacci, C., C. Barmo, G. Gallo, M. Maisano, T. Cappello, A. D'Agata, L. Claudio, M. Angela, F. Salvatore, C. Laura.** Effects of sublethal environmentally relevant concentrations of hexavalent chromium in the gills of *Mytilus galloprovincialis*. – *Aquat. Toxicol.*, **120-121**, 2012, 109-118.
5. **d'Isa, R., G. Comi, L. Leocani.** Apparatus design and behavioural testing protocol for the evaluation of spatial working memory in mice through the spontaneous alternation T-maze. – *Sci. Rep.*, **11**, 2021.
6. **Diallo, A., H. Jacobi, T. Schmitz-Hübsch, A. Cook, R. Labrum, A. Durr, A. Brice, P. Charles, C. Marelli, C. Mariotti, L. Nanetti, M. Panzeri, M. Rakowicz, A. Sobanska, A. Sulek, L. Schöls, H. Hengel, B. Melegh, A. Filla, A. Antenora, J. Infante, J. Berciano, B. P. van de Warrenburg, D. Timmann, S. Boesch, M. Pandolfo, J. B. Schulz, P. Bauer, P. Giunti, L. Baliko, M. H. Parkinson, J. Kang, T. Klockgether, S. T. du Montcel.** Body mass index decline is related to spinocerebellar ataxia disease progression. – *Mov. Discord. Clin. Pract.*, **4(5)**, 2017, 689-697.
7. **Dringen, R., J. M. Gutterer, J. Hirrlinger.** Glutathione metabolism in brain metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. – *Eur. J. Biochem.*, **267(16)**, 2009, 4912–4916.
8. **Faes, C., M. Aerts, H. Geys, L. De Schaepdrijver.** Modeling spatial learning in rats based on Morris water maze experiments. – *Pharm. Stat.*, **9(1)**, 2010, 10–20.
9. **Fahmy, A.** A histological study on the possible ameliorating effect of selenium on chromium (VI) induced neurotoxicity in the adult male Guinea pig cerebellar cortex. – *J. Am. Sci.*, **13(5)**, 2017, 8–17.
10. **Geethangili, M., S. Ding.** A review of the phytochemistry and pharmacology of *Phyllanthus urinaria* L. – *Front Pharmacol.*, **9**, 2018, 1109.
11. **Guerriero, R. M., C. C. Giza, A. Rotenberg.** Glutamate and GABA imbalance following traumatic brain injury. – *Curr. Neurol. Neurosci. Rep.*, **15(5)**, 2015, 27.
12. **Hao, P., Y. Zhu, S. Wang, H. Wan, P. Chen, Y. Wang, Z. Cheng, Y. Liu, J. Liu.** Selenium administration alleviates toxicity of chromium (VI) in the chicken brain. – *Biol. Trace. Elem. Res.*, **178(1)**, 2017, 127–135.
13. **Haraguchi, Y., Y. Mizoguchi, M. Ohgidani, Y. Imamura, T. Murakawa-Hirachi, H. Nabeta, H., H. Tateishi, T. A. Kato, A. Monji.** Donepezil suppresses intracellular Ca2+ mobilization through the PI3K pathway in rodent microglia. – *J. Neuroinflammation*, **14(1)**, 2017, 258.
14. **Hegazy, R., D. Mansour, A. Salama, A. Hassan, D. Saleh.** Exposure to intranasal chromium triggers dose and time-dependent behavioral and neurotoxicological defects in rats. – *Ecotoxicol. Environ. Saf.*, **216**, 2021, 112220.
15. **Igho, O., I. Afoke**. Histomorphological analysis of pyrethroid pesticide effects on the cerebrum and cerebellum in adult albino rats. – *J. Exp. Clin. Anat.*, **13(2)**, 2014, 54-59.
16. **Iteire, K. A., E. C. Osigwe, V. O. Ukwenya, F. U. Enemali, E. I. Odokuma.** *Carica Papaya* leaf crude-extract ameliorates lipopolysaccharide-induced neurotoxicity in mice cerebellum by downregulating GFAP and IBA-1 expression. – *J. Exp. Clin. Anat.*, **13(2)**, 2022, 275-288.
17. **Gawryluk, J. W., J. Wang, A. C. Andreazza, L. Shao, L. T. Young.** Decreased levels of glutathione, the major brain antioxidant, in post-mortem prefrontal cortex from patients with psychiatric disorders. – *Int. J. Neuropsychopharmacol.*, **14(1)**, 2011, 123–130.
18. **Klein, S.M, J. Vykoukal, P. Lechler, K. Zeitler, S. Gehmert, S. Schreml, E. Alt, U. Bogdahn, L. Prantl.** Noninvasive in vivo assessment of muscle impairment in the mdx mouse model – A comparison of two common wire hanging methods with two different results. – *J. Neurosci. Methods*, **203(2)**, 2012, 292–297.
19. **Kumar, K. B. H., R. Kuttan.** Protective effect of an extract of Phyllanthus amarus against radiation-indued damage in mice. – *J. Radiat. Res.*, **45(1)**, 2004, 133–139.

20. Li, S., L. Han, H. Shi, M. K. C. Chong, S. Zhao, J. Ran. Excess deaths from Alzheimer's and Parkinson's disease during the COVID-19 pandemic in the USA. – *Age Ageing*, **51(12)**, 2022, 1-6.
21. Merritt, K. P. McGuire, A. Egerton. Relationship between glutamate dysfunction and symptoms and cognitive function in psychosis. – *Front. Psychiatry*, **4(151)**, 2013, 1-8.
22. Musselman, K., C. T. Stoyanov, R. Marasigan, M. E. Jenkins, J. Konczak, S. M. Morton, A. J. Bastian. Prevalence of ataxia in children: a systematic review. – *Neurology*, **82(1)**, 2014, 80–89.
23. Nwankpa, P., M. U. Eteng, M. Akpamabiatu, G. Oze, H. Nwanjo. Effects of *Phyllanthus amarus* on serum lipid profile and oxidative stress status in Salmonellae typhi infected Wistar rats. – *J. Nat. Prod. Plant Resour.*, **2(5)**, 2012, 574–578.
24. Olopade, F. M. T. Shokunbi, A. Siren. Correlation of ventricular dilatation with neuropathological and neurobehavioral alterations in hydrocephalic rats. – *Fluids Barriers CNS*, **9(19)**, 2012, 1–10.
25. Omeiza, N. A., H. A. Abdulrahim, A. I. Alagbonsi, P. U. Ezurike, T. K. Soluoku, H. Isiabor, A. A. Alli-oluwafuyi. Melatonin salvages lead-induced neuro-cognitive shutdown, anxiety, and depressive-like symptoms via oxido-inflammatory and cholinergic mechanisms. – *Brain Behav.*, **11(8)**, 2021, e2227.
26. Owoeye, O., M. O. Gabriel. Protective effects of aqueous extract of Telfairia occidentalis on mercury-induced histological and oxidative changes in the rat hippocampus and cerebellum. – *Afr. J. Biomed. Res.*, **19(3)**, 2016, 241–247.
27. Oyeleye, S. I., T. A. Olasehinde, O. W. Fasakin, G. Oboh, J. A. Saliu. *Phyllanthus amarus* Schumach. & Thonn. and *Momordica charantia* L extracts improve memory function, attenuate cholinergic and purinergic dysfunction, and suppress oxidative stress in the brain of doxorubicin–treated rats. – *Phytomed. Plus.*, **2(2)**, 2022, 100283.
28. Patel, J., P. Tripathi, V. Shama, N. Chauhan, V. Dixit. Phyllanthus amarus: Ethnomedicinal uses, phytochemistry and pharmacology: a review. – *J. Ethnopharmacol.*, **138(2)**, 2011, 286–313.
29. Rao, S. K., C. Andrade, K. Reddy, K. N. Madappa, S. Thyagarajan, S. Chandra. Memory protective effect of indomethacin against electroconvulsive shock-induced retrograde amnesia in rats. – *Biol. Psychiatry*, **51(9)**, 2002, 770-773.
30. Rodriquez-Graña, T., R. Rodríguez-Labrada, S. Santana-Porbén, L. Reynaldo-Cejas, J. Medrano-Montero, N. Canales-Ochoa, Y. Silva-Ricardo, R. Torres-Vega, Y. González-Zaldivar, D. Almaguer-Gotay, G. Auburger, L. Velázquez-Pérez. Weight loss is correlated with disease severity in spinocerebellar ataxia type 2: a cross-sectional cohort study. – *Nutr. Neurosci.*, **25(8)**, 2022, 1747–1755.
31. Rotruck, J. T. A. L. Pope, H. E. Ganther, A. B. Swanson, D. G. Hafeman, W. G. Hoekstra. Selenium: biochemical role as a component of glutathione peroxidase. – *Science*, **179(4073)**, 1973, 588-590.
32. Seddik, L., T. M. Bah, A. Aoues, M. Brnderdour, M. Silmani. Dried leaf extract protects against lead-induced neurotoxicity in Wistar rats. – *Eur. J. Sci. Res.*, **42(1)**, 2010, 139-151.
33. Seibenhener, M. L., M. C. Wooten. Utilization of the open field maze for the assessment of locomotor and anxiety-like behavior in mice. – *J. Vis. Exp.,* **96**, 2015, 52434.
34. Shen, X. M., G. Dryhurst. Oxidation chemistry of (-)-norepinephrine in the presence of L-cysteine. – *J. Med. Chem.*, **39(10)**, 1996, 2018–2029.
35. Sian, J., D. T. Dexter, A. J. Lees, S. Daniel, Y, Agid, F. Javoy-Agid, P. Jenner, C. D. Marsden. Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. – *Ann. Neurol.*, **36(3)**, 1994, 348-355.
36. Singh, N. A., V. Bhardwaj, C. Ravi, N. Ramesh, A. K. A. Mandal, Z. A. Khan. EGCG nanoparticles attenuate aluminum chloride-induced neurobehavioral deficits, beta-amyloid, and tau pathology in a rat model of Alzheimer's disease. – *Front. Aging Neurosci.*, **10(244)**, 2018.

37. **Smeets, C. J. L. M., D. S. Verbeek.** Cerebellar ataxia and functional genomics: Identifying the routes to cerebellar neurodegeneration. – *Biochim. Biophys. Acta*, **1842(10)**, 2014, 2030–2038.
38. **Soudani, N., A. Troudi, I. B. Amara, H. Bouaziz, T. Boudawara, N. Zeghal.** Ameliorating effect of selenium on chromium (VI) induced oxidative damage in the brain of adult rats. – *J. Physiol. Biochem.*, **68(3)**, 2012, 397–409.
39. **Tobinick, E., H. Gross, A. Weinberger, H. Cohen.** TNF-alpha modulation for treatment of Alzheimer's disease: a 6-month pilot study. – *Med. Gen. Med.*, **8(2)**, 2006, 25.
40. **Veeresham, C.** Natural products derived from plants as a source of drugs. – *J. Adv. Pharm. Technol. Res.*, **3(4)**, 2012, 200–201.
41. **Vidal, J.** Consistent individual differences in some behaviors in mice of the C57Bl/6J inbred strain. – *UB. J. Psy.*, **46(2)**, 2016, 83–89.
42. **Wilkins, H. M., D. Kirchhof, E. Manning, J. W. Joseph, D. A. Linseman.** Mitochondrial glutathione transport is a key determinant of neuronal susceptibility to oxidative and nitrosative stress. – *J. Biol. Chem.*, **288(7)**, 2013, 5091-5101.
43. **Wise Jr, J. P., J. L. Young, J. Cai, L. Cai.** Current understanding of hexavalent chromium [Cr(VI)] neurotoxicity and new perspectives. – *Environ. Int.*, **158**, 2022, 106877.