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Alterations of Na^+/K^+-ATPase, cholinergic and antioxidant enzymes activity by protocatechuic acid in cadmium-induced neurotoxicity and oxidative stress in Wistar rats

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ABSTRACT

Background: This study assessed the possible protective mechanisms of protocatechuic acid (PCA) against cadmium (Cd)-induced oxidative stress and neurotoxicity in rats.
Methods: Male wistar strain rats weighing between 150–160 g were purchased and acclimatized for two weeks. The rats were divided into seven groups of seven each; NC group received normal saline, CAD group received 6 mg/kg of Cd-solution, CAD + PSG group received Cd-solution and prostigmine (5 mg/kg), CAD + PCA-10 and CAD + PCA-20 groups received Cd-solution and PCA (10 mg/kg and 20 mg/kg) respectively, PCA-10 and PCA-20 groups received 10 mg/kg and 20 mg/kg PCA each. Animals were administered normal saline, Cd and PCA daily by oral gavage for 21 days. After which the animals were sacrificed, the brain excised, homogenized and centrifuged. The activities of enzymes (Na^+/K^+-ATPase, cholinesterases, catalase, glutathione peroxidase, superoxide dismutase) and levels of oxidative stress markers (lipid peroxidation and reduced glutathione) linked to neurodegeneration were subsequently assessed.
Results: Significant ($p < 0.05$) alterations in the enzyme activities and levels of oxidative stress markers were observed in CAD group when compared to the NC group. However, the activities of the enzymes were reversed in CAD + PSG and CAD + PCA groups.
Conclusions: PCA may protect against cadmium-induced neurotoxicity by altering the activities of Na^+/K^+-ATPase, acetylcholinesterase, butyrylcholinesterase and endogenous antioxidant enzymes.

1. Introduction

Neurotoxicity occurs as a result of exposure to artificial or natural toxic substances (neurotoxic compounds) which alters the normal functioning of the nervous system in a way that oxidative injury or damage is inflicted on the nervous tissues [1]. This can on the long run disrupt or even destroy the vital cells responsible for the transmission and processing signals in the brain (neurons) and other regions of the nervous system leading to various neurological disorders [2]. Neurotoxicity can develop from exposure to heavy metals, radiation treatment, chemotherapy substances, drug therapies, certain foods and drug abuse as well as food additives and pesticides [3].

Cadmium (Cd) is a heavy metal and one of the most widely distributed toxic pollutants in the environment with a long biological half-life as a result of its low body excretion rate [4]. In view of this, over time, continuous vulnerability to Cd tend to lead to its accumulation thereby causing toxic effect in a variety of tissues [5]. The main source of exposure to cadmium is food such as grains and vegetables. Also, Cd can be taken or inhaled through the olfactory pathways or nasal mucosa into the central and peripheral neurons [6] which may alter the activities of the enzymes of the central nervous system such as cholinesterases. Cd induced toxicity is implicated in the production of reactive oxygen species (ROS) and exhaustion of antioxidants leading to oxidative stress in brain cells and alterations in the structural integrity of lipids [7] as well as neutralizing enzymes, which leads to the disturbances in

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brain metabolism [8]. The role of cadmium in neurological damage has been a target of research over the years and several researchers have shown that the effect of cadmium in neurological damage can be inhibited by some synthetic drugs, but this always comes with adverse side effects [9]. Also, some recent evidences have shown that plant phytochemicals such as polyphenols can inhibit the effects of cadmium neurotoxicity with little or no side effect [10].

Polyphenols are the mostly available antioxidants found in human diets. They are categorized in different classes as flavonoids, phenolic acids and lignans. Phenolic acids are naturally occurring compounds found in plants which are characterized with unique structural similarities like presence of carboxylic group as in protocatechuic, caffeic, *p*-coumaric, gallic, vanillic and ferulic acids, which exhibit antioxidant properties and contribute to the prevention of oxidative stress [11]. Recent findings by Oboh et al. [12] reported the anticholinesterase property of caffeic and chlorogenic acids (phenolic acids) in the management of Alzheimer's disease and oxidative stress. Protocatechuic acid (3,4-hydroxylbenzoic acid) is widely distributed in hundreds of plants as active phytoconstituent imparting various pharmacological potentials [13]. These effects may be attributed to its antioxidant activities, along with other possible mechanisms, such as interaction with several enzymes involved in the progression of some degenerative diseases.

Epidemiological and experimental evidences have affirmed that phenolic compounds may exert several biological effects such as antimicrobial, antihelminthic, antibacterial, anti-inflammatory, anti-diabetic, antioxidant and neuroprotective properties [14–17]. Although few studies have demonstrated hepatoprotective, antioxidative, nephroprotective, anti-cancer and neuroprotective potentials of PCA [18–21], however, the likely mechanisms of action of PCA in tissue and organ protection have not been fully addressed and investigated. Thus, this present study sought to investigate the possible neuroprotective mechanisms on cadmium-induced oxidative stress and neurotoxicity in rats' brain *in vivo* and antioxidant property of PCA as typified by its effect on antioxidant enzymes and lipid peroxidation.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals such as protocatechuic acid (PCA), cadmium, prostigmine, acetylthiocholine iodide, butyrylthiocholine iodide, adenosine triphosphate (ATP), ouabain, ammonium molybdate, thiobarbituric acid (TBA), dinitrophenylhydrazine (DNPH), sodium acetate, sodium dodecyl sulphate, phosphorus red, ascorbic acid, magnesium chloride, phosphate buffer of different molarity, adenosine monophosphate (AMP), sodium hydroxide, benzene, and 5,5′-dithio-bis(2-nitrobenzoic acid) were bought from Sigma-Aldrich, Chemie GmBH (Steinheim, Germany) while acetic acid was purchased from BDH Chemical Ltd., (Poole, England). The water used was glass distilled and all other chemicals and reagents used are of analytical grade.

2.2. Experimental animals and design

Forty-nine male Wistar rats of 160 g average weight were purchased from the Central Animal House, University of Ibadan, Ibadan, Oyo State Nigeria. The rats were acclimatized in stainless steel cages under controlled conditions of a 12 h light/dark cycle, 28 ° C temperature and 50% humidity for two weeks. The rats were allowed access to food and water ad libitum. There is average exposure to cadmium in the homes of some developing countries that derive their drinking water from ground, well and stream water located near industries that release cadmium as waste. Hence, we administered 6 mg/kg body weight of cadmium chloride orally to rats in this study to mimic average human cadmium exposure as previously reported [22–24]. The drug (prostigmine) and PCA used in this experiment were also administered orally.

2.3. Treatment groups

The animals were divided into seven groups of seven (7) rats each per steel cages and were treated for 21 days as follows:

NC- Wistar rats administered normal saline (Normal control).

CAD - Wistar rats administered 6 mg/kg body weight of cadmium only.

CAD + PSG- Wistar rats administered 6 mg/kg body weight of cadmium and treated with 5 mg/kg of prostigmine.

CAD + PCA-10 - Wistar rats administered 6 mg/kg body weight of cadmium and treated with 10 mg/kg body weight of PCA.

CAD + PCA-20 - Wistar rats administered 6 mg/kg body weight cadmium and treated with 20 mg/kg body weight of PCA.

PCA-10 - Wistar rats treated with 10 mg/kg body weight of PCA only.

PCA-20 - Wistar rats treated with 20 mg/kg body weight of PCA only.

2.4. Preparation of tissue homogenates

Rats decapitation were carried out via cervical dislocation while the cerebral tissue (whole brain) was rapidly dissected, weighed and placed in phosphate buffer pH 7.4 on ice. Subsequently, the tissue was rinsed with the phosphate buffer pH 7.4 and then homogenized with same buffer pH 7.4 (1:5 w/v), with about 10-up and down strokes at about 1200 rev/min in a Teflon-glass homogenizer (Pyrex Potter-Elvehjem model 7725T-45). The homogenate was centrifuged using microhematocrit centrifuge (LW Scientific M24) for 10 min at 3,000*g* to yield a pellet that was discarded and the supernatant was used for lipid peroxidation assay [25] as well as source of enzyme for the enzyme assays.

2.5. Experimental protocol

2.5.1. Na^+/K^+ –ATPase activity assay

The method described by Fiske and Subbarow. [26] was used to measure the activity of Na^+/K^+-ATPase in whole brain homogenate. Assay mixture consisted of 100 μL of Na^+, K^+ –ATPase substrate buffer (pH 7.4) (containing 120 mM Tris–HCl, 0.4 mM EDTA, 200 mM NaCl, 20 mM KCl, and 24 mM $MgCl_2$), 50 μL of supernatant (tissue homogenate) in the presence or absence of 50 μL of ouabain (1 mM), in a final volume of 200 μL. The reaction was initiated by the addition of 50 μL adenosine triphosphate (ATP). After incubating for 30 min at 37 °C, the reaction was terminated by the addition of 50 μL of 10% (w/v) trichloroacetic acid (TCA). The inorganic phosphate (Pi) released was quantified using a reaction mixture that contained 250 μL of ammonium molybdate (100 mM), 200 μL of reaction mixture from first grid, 500 μL of distilled water and 50 μL of ascorbic acid (8%). The absorbance was read at 620 nm using a UV/Visible spectrophotometer (Jenway Model No. 6315).

2.5.2. Acetylcholinesterase (AChE) assay

AChE activity investigation was carried out in a reaction mixture containing 50 μL of tissue homogenate, 50 μL of 5, 5′-dithiobis-(2-nitrobenzoic) acid (DTNB), 1175 μL of 0.1 M phosphate-buffered solution, pH 8.0). After incubation for 20 min at 25 °C, 25 μL of acetylthiocholine iodide solution was added as the substrate. The AChE activity was determined as changes in absorbance reading at 412 nm for 3 min at 25 °C [27,28] using a UV/Visible spectrophotometer (Jenway Model No. 6315).

2.5.3. Butyrylcholinesterase (BChE) assay

The modified method of Perry et al. [28] was used for the BChE activity investigation. This was determined in a reaction mixture containing 50 μL of tissue homogenate, 50 μL of 5, 5′-dithiobis (2-nitrobenzoic) acid (DTNB), 1175 μL of 0.1 M phosphate-buffered solution, pH 8.0. After incubation for 20 min at 25 °C, 25 μL butyrylthiocholine iodide solution was added as the substrate, and BChE activity was determined as changes in absorbance reading at 412 nm for 3 min at 25 °C using a UV/Visible spectrophotometer (Jenway Model No. 6315).

2.5.4. Lipid peroxidation and thiobarbituric acid reactions

300 μL of the tissue homogenate was pipette into fresh test tubes. Subsequently, 300 μL of 8.1% sodium dodecyl sulphate (SDS), 500 μL of acetic acid/HCl buffer (pH 3.4) and 500 μL of 0.6% thiobarbituric acid (TBA) were added to the test tubes. This mixture was incubated at 100 °C for 1 h and TBA reactive species (TBARS) produced were measured at 532 nm using a UV/Visible spectrophotometer (Jenway Model No. 6315). Malondialdehyde (MDA) was used as standard and TBARS produced was reported as MDA equivalent [29]. The lipid peroxidation value was calculated.

2.5.5. Determination of catalase activity

Briefly, 50 μL of the test sample was added to a reacting mixture containing 500 μL of 59 mM H_2O_2 and 950 μL of 50 mM phosphate buffer (pH 7.0). The reaction was carried out at 25 °C and the decrease in absorbance at 240 nm was monitored for 3 min at 15 s interval. A unit of the enzyme activity is defined as the amount of enzyme catalyzing the decomposition of 1 μmol of hydrogen peroxide per minute at 25 °C and pH 7.0 [30].

2.5.6. Determination of reduced glutathione (GSH) content

1 mL of supernatant was treated with 500 μL of Ellman's reagent (19.8 mg of 5,5′-dithiobisnitrobenzoic acid in 100 mL of 0.1% sodium citrate) and 3.0 mL of 0.2 M phosphate buffer (pH 8.0) [31]. The absorbance was read at 412 nm in UV/Visible spectrophotometer (Jenway Model No. 6315).

2.5.7. Determination of glutathione peroxidase (GSH-Px) activity

Tissue homogenate was obtained from supernatant after centrifuging 5% brain homogenate at 15000 × g for 10 min followed by 10000 × g for 30 min at 4 °C was used. One milliliter of reaction mixture was prepared which contained 0.3 mL of phosphate buffer (0.1 M, pH = 7.4). 0.2 mL of 2 mM GSH, 0.1 mL of sodium azide (10 mM), 0.1H_2O_2 (1 mM) and 0.3 mL of tissue homogenate. After incubation at 37 °C for 15 min, the reaction was terminated by addition of 0.5 mL of 5% TCA. Tubes were centrifuged at 1500 × *g* for 5 min and the supernatant was collected. 0.2 mL of phosphate buffer (0.1 M, pH = 7.4) and 0.7 mL of DTNB (0.4 mg/mL) were added to 0.1 mL of reaction supernatant. After mixing, absorbance was recorded at 420 nm [32] using UV/Visible spectrophotometer (Jenway Model No. 6315).

2.5.8. Determination of superoxide dismutase (SOD) activity

Briefly, 0.1 mL of the tissue homogenate was diluted in 0.9 mL of distilled water to make 1 in 10 dilutions. An aliquot of 0.2 mL of the diluted microsome was added to 2.5 mL of 0.05 M carbonate buffer pH 10.2 to equilibrate in a cuvette and the reaction started by the addition 0.3 mL of 0.3 M of adrenaline. The reference cuvette contained 2.5 mL of carbonate buffer, 0.3 mL of substrate (adrenaline) and 0.2 mL of distilled water. The increase in absorbance at 480 nm was monitored every 30 s for 150 s [33].

2.5.9. Histological assessment

Drury and Wallington. [34] method was used for the histological assessment. Rats brains from different groups were inserted in 10% neutral formalin solution, dehydrated in absolute ethanol and embedded in paraffin. Fine sections obtained were mounted on glass slides and counter-stained with hematoxylin and eosin (H&E) for light microscopic analyses. Photographs were subsequently taken.

2.6. Data analysis

Results of replicate readings were pooled and expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to analyze the results and turkey test was used for the post hoc treatment [35]. Graph Pad prism 6.0 software package for Windows was used for the analysis.

3. Results

3.1. Na^+/K^+-ATPase activity

In this study, the Na^+/K^+ ATPase activity was significantly ($P < 0.05$) reduced in CAD group when compared to the NC group (Fig. 1). Moreover, the Na^+/K^+ ATPase activity was significantly ($P<0.05$) elevated in CAD + PSG, CAD + PCA-10 and CAD + PCA-20 groups. In addition, there was no significant ($P>0.05$) difference between the PCA-10 group and NC group

3.2. Acetylcholinesterase and butyrylcholinesterase activity

Further investigation in this study showed that there was a significant ($P < 0.05$) elevation in AChE activity of rats in CAD group when compared to the NC group (Fig. 2). However, the AChE activity was significantly ($P<0.05$) reduced in CAD + PSG, CAD + PCA-10 and CAD + PCA-20 when compared to the CAD group. In addition, there was no significant ($P>0.05$) difference between PCA-10 and PCA-20 groups when compared with NC group (Fig. 2).

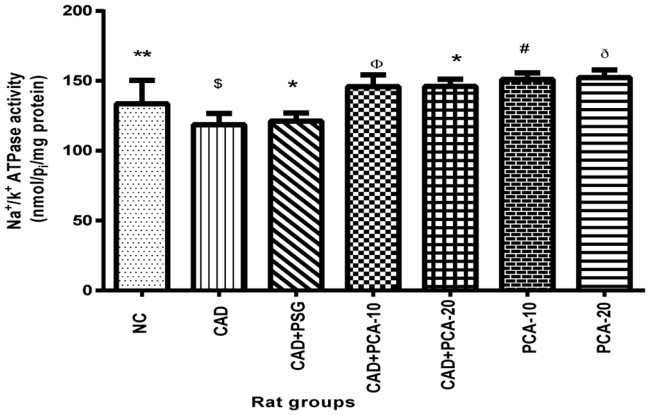

Fig. 1. Effects of PCA on Na^+/K^+ ATPase activity in cadmium-induced neurotoxicity in rats. Values represent mean ± standard deviation (n = 7).
Keys: NC group – normal control rats, treated with normal saline; CAD group – rats treated with 6 mg/kg body weight cadmium only; CAD + PSG group – rats treated with 6 mg/kg body weight cadmium and prostigmine; CAD + PCA-10 group – rats treated with 6 mg/kg body weight cadmium and 10 mg/kg body weight of protocatechuic acid (PCA); CAD + PCA-20 group – rats treated with 6 mg/kg body weight cadmium and 20 mg/kg body weight of protocatechuic acid (PCA); PCA-10 group; normal rats treated with 10 mg/kg body weight protocatechuic acid (PCA); PCA-20 group- normal rats treated with 20 mg/kg body weight protocatechuic acid (PCA).
$ - CAD group was significantly ($P < 0.05$) different from NC group.
* - CAD + PSG and CAD + PCA-10 groups were significantly ($P < 0.05$) different from CAD group.
** - CAD + PCA-20 group was significantly ($P < 0.05$) different from CAD group
ð - PCA-10 group was not significantly ($P > 0.05$) different from NC group.
- PCA-20 was significantly ($P < 0.05$) different from NC.

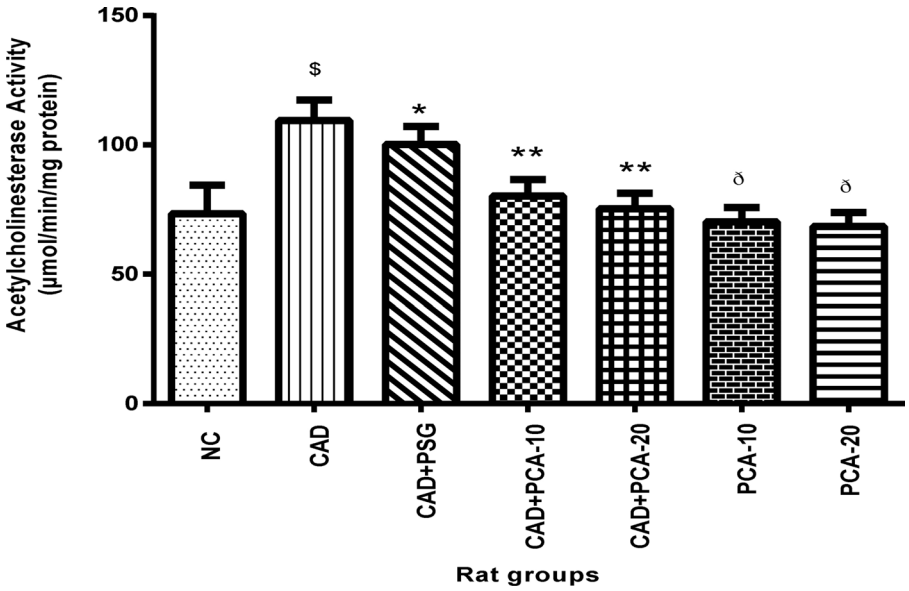

Fig. 2. Effects of PCA on acetylcholinesterase (AChE) activity in cadmium-induced neurotoxicity in rats' brain. Values represent mean ± standard deviation (n = 7).
Keys: NC group – normal control rats, treated with normal saline; CAD group – rats treated with 6 mg/kg body weight cadmium only; CAD + PSG group – rats treated with 6 mg/kg body weight cadmium and prostigmine; CAD + PCA-10 group – rats treated with 6 mg/kg body weight cadmium and 10 mg/kg body weight of protocatechuic acid (PCA); CAD + PCA-20 group – rats treated with 6 mg/kg body weight cadmium and 20 mg/kg body weight of protocatechuic acid (PCA); PCA-10 group; normal rats treated with 10 mg/kg body weight protocatechuic acid (PCA); PCA-20 group- normal rats treated with 20 mg/kg body weight protocatechuic acid (PCA).
$ - CAD group was significantly ($P < 0.05$) different from NC group.
* - CAD + PSG group was significantly ($P < 0.05$) different from CAD group.
** - CAD + PCA-10 and CAD + PCA-20 groups were very significantly ($P < 0.05$) different from CAD group.
ð - PCA-10 and PCA-20 groups were not significantly ($P > 0.05$) different from NC group.

In like manner, the BChE activity was significantly ($p < 0.05$) elevated in CAD group ($P < 0.05$) when compared to the NC group. However, rat administered cadmium and treated with prostigmine (a synthetic drug) and doses of PCA (10 – 20 mg/kg) had significantly ($P < 0.05$) reduced BChE activity (Fig. 3).

3.3. Lipid peroxidation and antioxidant enzymes

The effect of PCA on cadmium-induced toxicity in rat brain investigated revealed that the MDA level was significantly ($P < 0.05$) elevated in CAD group when compared to the NC group. Moreover, there was significant ($P < 0.05$) reduction in MDA level in the animal groups treated with both cadmium and either prostigmine or 10 or 20 mg/kg of PCA (Fig. 4). In addition, the influence of PCA on catalase activity was investigated and the result presented in Fig. 5. A dramatic reduction in catalase enzyme activity was observed in rats administered cadmium compared to the normal control group. However, in cadmium induced neurotoxicity groups that were treated with prostigmine and either 10 or 20 mg/kg PCA showed significant elevation ($P<0.05$) while catalase activity in PCA-10 and PCA-20 groups were not significantly ($P>0.05$) different from NC group.

Fig. 7 shows the effect of prostigmine and PCA on glutathione peroxidase (GSH-Px) in the presence of cadmium. The phenolic compound increased the enzyme activity, which otherwise decreased dramatically in CAD group. There was no significant ($P > 0.05$) difference between PCA-20 and NC group.

3.4. Histological examination

Figs. 9 showed the histopathological examination of the rats' brain sections. Normal rats showed normal mild neuroglia architecture; no significant lesions were observed (Fig. 9, Plate 1). In CAD group, the brain showed mild congestion of the meninges and mild lesions (Fig. 9, Plate 2). The brain of rats treated

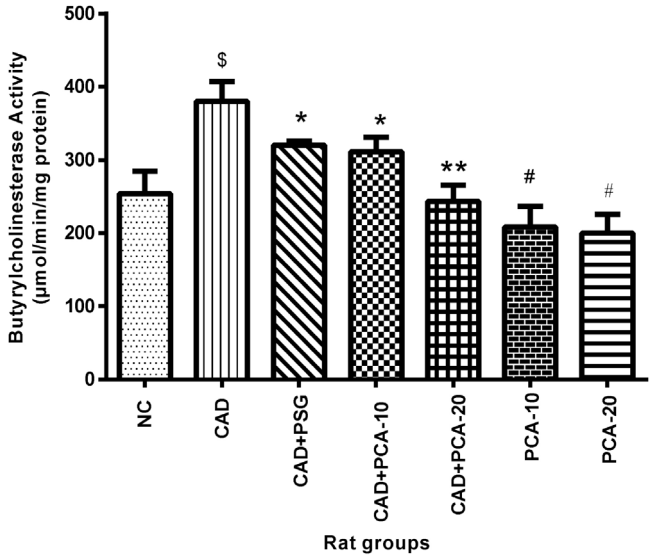

Fig. 3. Effects of PCA on butyrylcholinesterase activity (BChE) in cadmium-induced neurotoxicity in rats. Values represent mean ± standard deviation (n = 7).
Keys: NC group – normal control rats, treated with normal saline; CAD group – rats treated with 6 mg/kg body weight cadmium only; CAD + PSG group – rats treated with 6 mg/kg body weight cadmium and prostigmine; CAD + PCA-10 group – rats treated with 6 mg/kg body weight cadmium and 10 mg/kg body weight of protocatechuic acid (PCA); CAD + PCA-20 group – rats treated with 6 mg/kg body weight cadmium and 20 mg/kg body weight of protocatechuic acid (PCA); PCA-10 group; normal rats treated with 10 mg/kg body weight protocatechuic acid (PCA); PCA-20 group- normal rats treated with 20 mg/kg body weight protocatechuic acid (PCA).
$ - CAD group was significantly ($P < 0.05$) different from NC group.
* - CAD + PSG and CAD + PCA-10 groups were significantly ($P < 0.05$) different from CAD group.
** - CAD + PCA-20 group was very significantly ($P < 0.05$) different from CAD group.
# - PCA-10 and PCA-20 groups were significantly ($P < 0.05$) different from NC group.

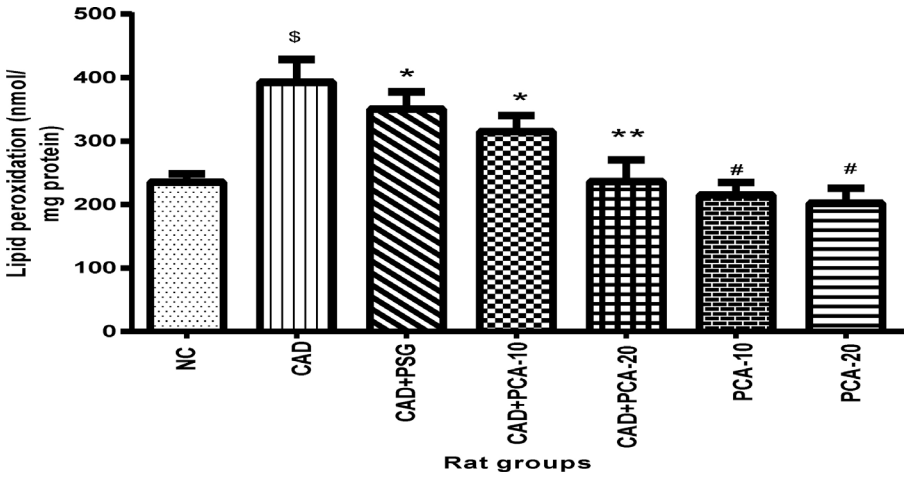

Fig. 4. Effects of PCA on lipid peroxidation (LPO) in cadmium-induced oxidative stress in rats' brain. Values represent mean ± standard deviation (n = 7).
Keys: NC group – normal control rats, treated with normal saline; CAD group – rats treated with 6 mg/kg body weight cadmium only; CAD + PSG group – rats treated with 6 mg/kg body weight cadmium and prostigmine; CAD + PCA-10 group – rats treated with 6 mg/kg body weight cadmium and 10 mg/kg body weight of protocatechuic acid (PCA); CAD + PCA-20 group – rats treated with 6 mg/kg body weight cadmium and 20 mg/kg body weight of protocatechuic acid (PCA); PCA-10 group; normal rats treated with 10 mg/kg body weight protocatechuic acid (PCA); PCA-20 group- normal rats treated with 20 mg/kg body weight protocatechuic acid (PCA).
$ - CAD group was significantly ($P < 0.05$) different from NC group.
* - CAD + PSG and CAD + PCA-10 groups were significantly ($P < 0.05$) different from CAD group
** - CAD + PCA-20 group was very significantly ($P < 0.05$) different from CAD group.
# - PCA-10 group and PCA-20 were significantly ($P < 0.05$) different from NC group.

with oral administration of 6 mg/kg and 5 mg/kg body weights per day of Cd and prostigmine respectively had no significant lesions (Fig. 9, Plate 3). Co-administration of Cd and 10 mg/kg of protocatechuic acid; showed no significant lesion (Fig. 9, Plate 4). Likewise, co-administration of Cd and 20 mg/kg of protocatechuic acid; showed no significant lesion (Fig. 9, Plate 5). As observed in Plate 6 and 7 of Fig. 9, administration of 10 and 20 mg/kg of PCA acid per day respectively to rats revealed there were no lesions.

4. Discussion

Neuronal damage may be caused by the free radicals' production by cadmium which may alter the activities of key enzymes of the nervous system [36]. Cadmium (Cd) has also been shown to generate free radicals in the brain and threaten both the neurons and glial cells of the CNS [37]. Consumption of diet rich in polyphenolic compounds is speculated to be a dietary strategy towards the prevention, treatment and/or management of several

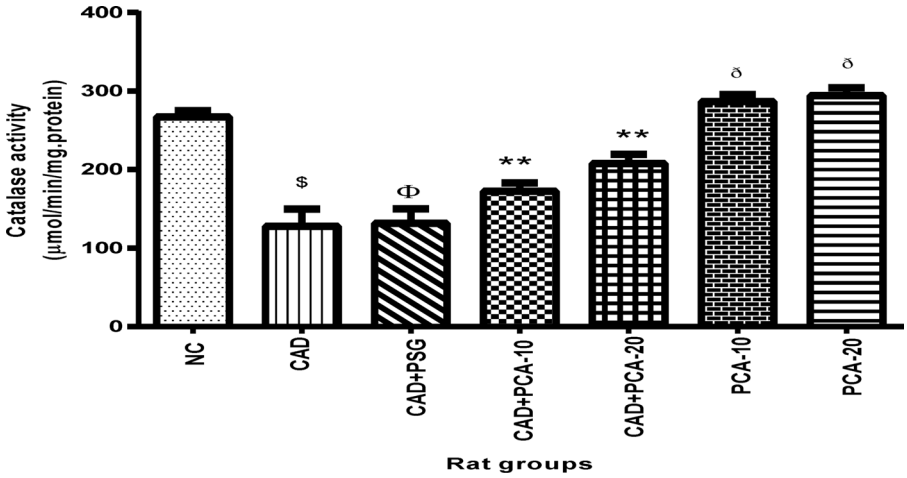

Fig. 5. Effects of PCA on catalase activity in cadmium-induced oxidative stress in rats' brain. Values represent mean ± standard deviation (n = 7).
Keys: NC group – normal control rats, treated with normal saline; CAD group – rats treated with 6 mg/kg body weight cadmium only; CAD + PSG group – rats treated with 6 mg/kg body weight cadmium and prostigmine; CAD + PCA-10 group – rats treated with 6 mg/kg body weight cadmium and 10 mg/kg body weight of protocatechuic acid (PCA); CAD + PCA-20 group – rats treated with 6 mg/kg body weight cadmium and 20 mg/kg body weight of protocatechuic acid (PCA); PCA-10 group; normal rats treated with 10 mg/kg body weight protocatechuic acid (PCA); PCA-20 group- normal rats treated with 20 mg/kg body weight protocatechuic acid (PCA).
$ - CAD group was significantly ($P < 0.05$) different from NC group.
Φ - CAD + PSG group was not significantly ($P > 0.05$) different from CAD group.
** - CAD + PCA-10 and CAD + PCA-20 groups were very significantly ($P < 0.05$) different from CAD group.
ð - PCA-10 and PCA-20 groups were not significantly ($P < 0.05$) different from NC group.

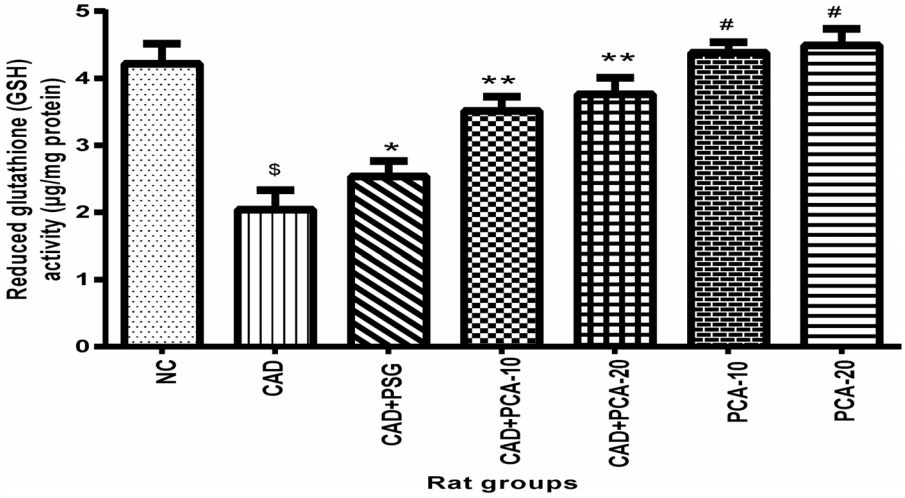

Fig. 6. Effects of PCA on reduced glutathione (GSH) activity in cadmium-induced oxidative stress in rats' brain. Values represent mean ± standard deviation (n = 7).
Keys: NC group – normal control rats, treated with normal saline; CAD group – rats treated with 6 mg/kg body weight cadmium only; CAD + PSG group – rats treated with 6 mg/kg body weight cadmium and prostigmine; CAD + PCA-10 group – rats treated with 6 mg/kg body weight cadmium and 10 mg/kg body weight of protocatechuic acid (PCA); CAD + PCA-20 group – rats treated with 6 mg/kg body weight cadmium and 20 mg/kg body weight of protocatechuic acid (PCA); PCA-10 group; normal rats treated with 10 mg/kg body weight protocatechuic acid (PCA); PCA-20 group- normal rats treated with 20 mg/kg body weight protocatechuic acid (PCA).
$ - CAD group was significantly ($P < 0.05$) different from NC group.
* - CAD + PSG group was not significantly ($P > 0.05$) different from CAD group.
** - CAD + PCA-10 and CAD + PCA-20 groups were very significantly ($P < 0.05$) different from CAD group.
# - PCA-10 and PCA-20 groups were significantly ($P < 0.05$) different from NC group.

neurodegenerative diseases [38,39]. PCA is widely found in nature most especially in edible plants such as grains, vegetables, cereals and fruits in human diet are used in folk medicine for disease management [10,11]. PCA was reported to possess various pharmacological potentials such as neuroprotective effects which is adjudged to be closely correlated with its antioxidant activities [19–21,40]. The present study investigated the possible PCA mechanisms of action in neuronal damage induced by cadmium chloride.

Na^+/K^+-ATPase is identified as a key transmembrane enzyme of the central nervous system vital for regulation of intracellular pH, cell volume and calcium concentration, as well as for exchange of solutes transported by coupled systems. Excessive reduction in Na^+/K^+-ATPase activity may have been associated with neuronal damage caused by excess ROS generated as a result of cadmium induction. As observed in Fig. 1, the decreased activity of Na^+/K^+-ATPase in CAD group when compared to NC group, may be an indication of neuronal damage which may be linked with memory loss and deficit in cognitive function [41]. However, treatment with 10 and 20 mg/ kg body weight of PCA increased the activity of the enzyme in rats administered cadmium (Fig. 1). The result agrees with earlier study by Silva et al. [42] where a traumatic brain injury was reported, following a decrease in Na^+/K^+-ATPase activity in the brain and concomitant increase in lipid and protein oxidation levels. In another study, neuroprotective role of curcumin was reported and linked with the elevation in the activity of Na^+/K^+-ATPase in regions of rats' brain [43]. In a similar manner, Rajagopal et al. [44] reported the elevation of Na^+/K^+- ATPase activity by phenolic-rich *Ocimum sanctum* leaf extract after excitotoxicity induced by monosodium glutamate. From this study, PCA altered neurotoxicity as substantiated by its ability to increase Na^+/K^+-ATPase activity after a reduced activity as a result of cadmium induced neurotoxicity.

Oxidative stress implicated by free radicals has been implicated in cholinergic system impairment, resulting to various neurodegenerative conditions [45]. Inhibitions of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities have been shown to be a practical strategy to minimize the hydrolysis of acetylcholine and therapeutic targets for Alzheimer's disease management [46]. The observed increase in AChE activity in CAD

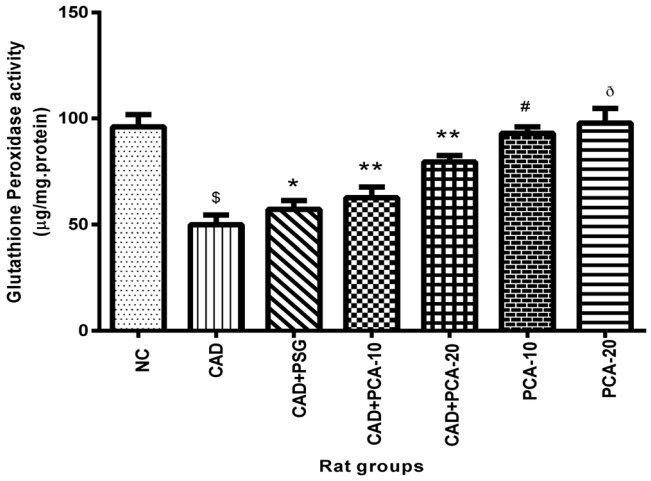

Fig. 7. Effects of PCA on glutathione peroxidase (GSH-Px) activity in cadmium-induced oxidative stress in rats' brain. Values represent mean ± standard deviation (n = 7).
Keys: NC group – normal control rats, treated with normal saline; CAD group – rats treated with 6 mg/kg body weight cadmium only; CAD + PSG group – rats treated with 6 mg/kg body weight cadmium and prostigmine; CAD + PCA-10 group – rats treated with 6 mg/kg body weight cadmium and 10 mg/kg body weight of protocatechuic acid (PCA); CAD + PCA-20 group – rats treated with 6 mg/kg body weight cadmium and 20 mg/kg body weight of protocatechuic acid (PCA); PCA-10 group; normal rats treated with 10 mg/kg body weight protocatechuic acid (PCA); PCA-20 group- normal rats treated with 20 mg/kg body weight protocatechuic acid (PCA).
$ - CAD group was significantly ($P < 0.05$) different from NC group.
* - CAD + PSG group was significantly ($P < 0.05$) different from CAD group.
** - CAD + PCA-10 and CAD + PCA-20 groups were very significantly ($P < 0.05$) different from CAD group.
# - PCA-10 group was significantly ($P < 0.05$) different from NC group.
ð - PCA-20 group was not significantly ($P > 0.05$) different from NC group.

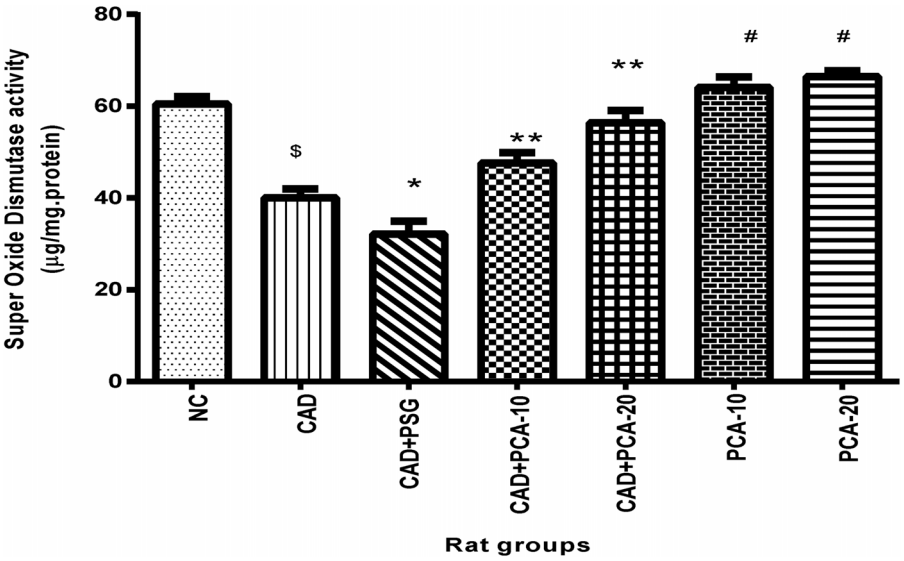

Fig. 8. Effects of PCA on superoxide dismutase (SOD) activity in cadmium-induced oxidative stress in rats' brain. Values represent mean ± standard deviation (n = 7).
Keys: NC group – normal control rats, treated with normal saline; CAD group – rats treated with 6 mg/kg body weight cadmium only; CAD + PSG group – rats treated with 6 mg/kg body weight cadmium and prostigmine; CAD + PCA-10 group – rats treated with 6 mg/kg body weight cadmium and 10 mg/kg body weight of protocatechuic acid (PCA); CAD + PCA-20 group – rats treated with 6 mg/kg body weight cadmium and 20 mg/kg body weight of protocatechuic acid (PCA); PCA-10 group; normal rats treated with 10 mg/kg body weight protocatechuic acid (PCA); PCA-20 group- normal rats treated with 20 mg/kg body weight protocatechuic acid (PCA).
$ - CAD group was significantly ($P < 0.05$) different from NC group.
* - CAD + PSG was significantly ($P < 0.05$) different from CAD group.
** - CAD + PCA-10 and CAD + PCA-20 groups were very significantly ($P < 0.05$) different from CAD group.
# - PCA-10 and PCA-20 groups were significantly ($P < 0.05$) different from NC group.

rat group may suggest the neurotoxic effect of cadmium at the dose administered. However, significant ($p < 0.05$) reduction was observed in rats treated with PCA as shown in Fig. 2. This is in accordance with the earlier findings of Szwajgier and Borowiec [47] where phenolic acids characterized from extracts of selected fruits and vegetables were shown to inhibit acetylcholinesterase *in vitro*. Also, Yash et al. [48] showed the reduction in acetylcholinesterase activity by PCA and reported that it ameliorated scopolamine induced amnesia in rats *in vivo*. The non-significant ($p > 0.05$) difference between the normal rats treated with PCA and the control rats may be an indication of the safety of the different doses of PCA. The inhibition of the cholinesterases activities elevated by cadmium suggests the neuroprotective role of PCA upon cadmium exposure. Therefore, collective inhibition of the activities of the cholinesterases has been described to boost brain cholinergic function and amend memory deficit associated with AD [46]. Aside the cholinergic function, attenuating oxidative damage may be another therapeutic approach by the use of antioxidative agents in neuronal management.

Lipid peroxidation is a significant common cellular process oxidative stress conditions. It involves the formation of peroxyl radicals that can rearrange by the process of cyclisation to form endoperoxides [49,50]. The endoperoxides so formed can disintegrate to form series of secondary products like a highly reactive malondialdehyde which can react with biological substrates like proteins and DNA [49]. The link between lipid peroxidation and ROS was established in the report of Shukla and Chandra [51] that malondialdehyde is a pointer of lipid peroxidation and its attendant oxidative stress. The elevation of MDA level in rats administered cadmium in this study may be linked to the oxidative stress resulting from deleterious effect of metal-induced ROS on polyunsaturated fatty acid residues of phospholipids [52]. A slight reduction observed in the MDA levels of the PCA treated rats when compared with the normal control rats could be likened to the findings of Lende et al. [53] where PCA reduced the lipid peroxidation level in mice suggesting that PCA may exert modulatory effect on excessive ROS generated in the brain as a result of exposure to cadmium.

The reduction in the catalase activity observed in the brain of rats exposed to cadmium as seen in Fig. 5, may be attributed to metal deficiency as it is established that cadmium reduces iron levels in tissues [54], the decrease in iron levels might result in reduction in catalase activity as iron is identified as a major component of catalase active site. Similarly, drastic reduction in reduced glutathione (GSH) level in the brain of cadmium-induced neurotoxicity group when compared with the normal control was observed. This reduced level was restored towards normalization in CAD + PSG, CAD + PCA-10 and CAD + PCA-20 groups while GSH levels in PCA-10 and PCA-20 were significantly ($P < 0.05$) different from the NC group (Fig. 6). Depletion of GSH level of rat brain exposed to cadmium (Fig. 6) might be as a result of heavy metals toxicity which occurs when high metal affinity sulfhydryl group of cysteine moiety of glutathione forms strong mercaptide complexes that are thermo-dynamically stable with cadmium. The decreased GSH level in cadmium treated animals suggests a possible metal binding to the enzyme active site or an interaction between the active amino acids of this enzymes and accumulated free radicals [55,56]. Also, oxidation of GSH may occur as result of interaction with the free radicals released by metals which may in turn lead to consumption of GSH level during metal detoxification as previously reported by Manna et al. [56]. Similarly, a substantial reduction ($P < 0.05$) in superoxide dismutase activity in CAD group compared to the NC group. It is worth noting that the results of catalase (Fig. 5), GPx (Fig. 6) and SOD (Fig. 8) activities showed similar trend and this may indicate that antioxidant enzyme activities are altered by cadmium toxication in the rats. Superoxide dismutase, a vital defense enzyme converts oxygen to hydrogen peroxide and thus plays a vital role in protecting against damage induced by superoxide accumulation in physiological system [57].

From this study, we suspect that cadmium inhibits many enzymes activity by virtue of its entry into the mitochondria thereby inhibiting protein synthesis or binding to their thiol

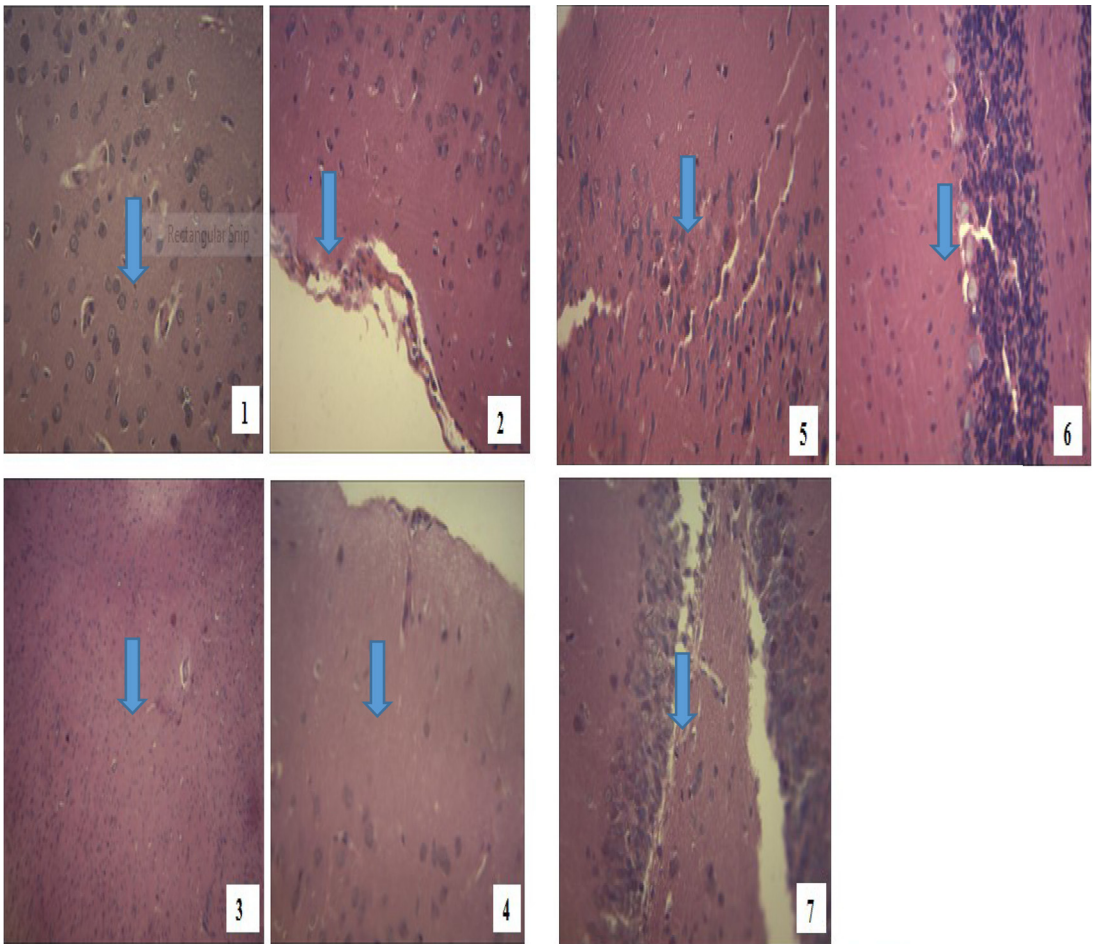

Fig. 9. Histopathological examination of the brain sections from: normal control rats, treated with normal saline (Plate 1); rats treated with 6 mg/kg body weight cadmium only (Plate 2); rats treated with 6 mg/kg body weight cadmium and prostigmine (Plate 3); rats treated with 6 mg/kg body weight cadmium and 10 mg/kg body weight protocatechuic acid (Plate 4); rats treated with 6 mg/kg body weight cadmium and 20 mg/kg body weight protocatechuic acid (Plate 5); normal rats treated with 10 mg/kg body weight protocatechuic acid (Plate 6); normal rats treated with 20 mg/kg body weight protocatechuic acid (Plate 7).

groups thereby leading to increased antioxidant enzyme levels [58]. We suspect that the binding of cadmium to the cysteine moiety in reduced glutathione results in the inactivation of glutathione peroxidase, which, eventually, stall the conversion of hydrogen peroxide to water [59]. Thus, the observed regularization of these antioxidant enzymes activity in this study due to PCA treatment could be adjudged as its vital potential to reduce the levels and proliferation of reactive oxygen species and thus development of tissue injury.

A section of this study which examined the histology of the rats' brain tissues revealed that the intervention of PCA ameliorated the toxic effect of Cd in rats' brain. This also indicate the ability of PCA to reduce degenerative changes that could result from neuronal disorders. Wolfe and Molinoff [60] suggested brain degenerative changes that could lead to Alzheimer's disease in people exposed to Cd. Similarly, Sidman and Leviton [61] concluded that Cd might induce untimely aging process. Findings from this study is in agreement with the one recorded by Dzobo and Naik [62] who observed a lowering effect of selenium on cadmium induced elevated esterase activity and oxidative stress in rat organs. This confirms the neuroprotective potential of PCA and safety in the dose administered in this study which is within the obtainable quantity from plant foods rich in this phenolic compound. The possible mechanism of action of PCA against cadmium induced neurotoxicity may be involved in the regulation of the activities of Na^+/K^+-ATPase and cholinesterases with attenuation of antioxidant status relevant to neurological diseases.

5. Conclusion

We conclude from this study that cadmium may induce neurotoxicity in rats through the free radical mediated oxidative stress and activation/inactivation of key enzymes. However, part of the possible mechanism of neuroprotection of PCA may involve: reduction in lipid peroxidation level and regulation of brain antioxidant enzyme activities as well as the regulation of cholinergic and Na^+/K^+- ATPase activities.

Compliance with ethical standards, acknowledgement and conflict of interest

The animals used received humane care and the ethical standards and regulations have been followed in accordance with national and institutional guidelines for the protection of animals' welfare during experiments. Human participants were not used in this study and the whole experiment was carried out at the Functional Foods, Nutraceuticals and Phytomedicine Unit Laboratory of the Department of Biochemistry, Federal University of

Technology, Akure, Ondo State, Nigeria. The authors received no financial support or funding of any sort from any agency, organisation or institution as this whole research was solely funded by the authors and also declare no conflict of interest as far as this manuscript is concerned.

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