

Modulatory effect of fermented *Tetracarpidium conophorum* (African walnut) supplemented diet on cadmium-induced toxicity in rats

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Abstract

Tetracarpidium conophorum (African Walnut) is a plant with acclaimed multi-therapeutic properties in different parts of the plant. This research investigated the effect of fermented walnut supplemented diet on cadmium-induced toxicity in the liver and brain of rats. Twenty male Wistar rats were divided into four groups of five animals each weighing between 90-140 g. Group 1 received 5 mg/kg body weight cadmium chloride (CdCl₂) and normal rat feed. Group 2 received a normal rat diet while groups 3 and 4 received 5 mg/kg body weight of cadmium chloride, and 5% and 10% walnut supplemented feed respectively. Cadmium (Cd) was administered daily for 6 weeks by oral intubation. Rats were sacrificed 24 hrs after the final treatment. Cd exposure elicited increased activities of Acetylcholinesterase, Superoxide dismutase, Catalase as well as elevated Glutathione levels. In addition, Cd exposure caused increases in rat plasma cholesterol and triglyceride concentration. The fermented walnut supplemented diet restored some rats' biochemical parameter to near normal comparable to control. Our study shows that walnut supplemented food could substantially moderate Cd-induced toxicity in rat liver and brain while providing health and nutritional benefits. Hence, it could be useful for occupationally exposed individuals as a dietary intervention to reduce adverse health effects.

1. Introduction

Heavy metals among several environmental pollutants pose an insidious threat and have been gaining global attention in recent decades probably because of the phenomenal increase in population, economic development and anthropogenic activities in the industrial, agricultural and mining sectors (World Health Organization, 2019; Nasrollahi *et al.*, 2020). Chemical pollutants released into different environmental matrices (water, soil and air) eventually reach the food chain from the environment and may eventually bio-accumulate in organisms.

Cadmium (Cd) is one of the heavy metals that pose considerable environmental and occupational concerns. It is versatile and therefore applied for several industrial processes as a corrosion inhibitor, production of Cd

batteries, as colour pigment and as neutron generator in nuclear power stations (Godt *et al.*, 2006). Human exposure to Cd, particularly among occupationally exposed employees, may present serious health challenges (Alli, 2019).

Previous studies reported that Cd triggers oxidative stress with quantitative abatement of antioxidant enzymes such as glutathione-S-transferase, superoxide dismutase and catalase in the central nervous system (Shagirtha *et al.*, 2011). This supposed response of antioxidant enzymes is not sacrosanct as a few studies have reported a decline in the activities of these antioxidant enzymes in the brain on exposure to Cd (Shukla *et al.*, 1996; Nemmiche *et al.*, 2007; Zhang *et al.*, 2009), suggesting that the fundamental basis for oxidative stress triggered by Cd, cellular response and

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supposed functions is unpredictable. Cadmium causes an adverse effect on thiol status in the body. Cadmium has a high affinity for thiol in cysteine-rich metallothionein which is known to sequester heavy metals (Nishitai and Matsuoka, 2008), thus leading to oxidative stress.

Due to the adverse effect and cost of conventional orthodox pharmaceuticals in the treatment and management of diseases, there is a drive towards exploring the potential of natural product resource for nutritional and medicinal purposes. Consequently, investigations targeted at identifying natural food substances that can be used effectively to manage heavy metal toxicity with minimal side effects are welcome. A variety of plants such as garlic, ginger, onions, kola nut and green tea have been used to treat Cd and lead toxicity by way of dietary intervention (Zhai et al., 2015; Osemwegie et al., 2017).

The African walnut, *Tetracarpidium conophorum* has been reported as one of such plants. The walnut is a member of the Euphorbiaceae family. It is a climbing plant distributed in Southern Nigeria and the West African sub-region. It has a wrinkled two-lobed seed that is white in colour enclosed in a dark brown and hard shell. African walnut is a robust repository of protein, vitamins, polyphenol and antioxidants; and its oil is abundant in oleic acid, stearic acids, linolenic acid and mono-saturated fatty acids (Kanu et al., 2015). Many studies have ascertained the antioxidant (Amaeze et al., 2011), anticancer (Carvalho et al., 2010), chelating (Olabinri et al., 2010), antidiabetic (Odoemena et al., 2010), antiplasmodial (Dada and Ogundolie, 2016) and antilipidemic (Ezealisiji et al., 2016) properties of different parts of this tropical plant. Other documented benefits are the amelioration of reproductive toxicity (Akomolafe et al., 2015) and erectile dysfunction (Akomolafe et al., 2017). In light of the foregoing, rat animal model was used to evaluate the effect of fermented walnut-supplemented feed for protection against Cd-induced toxicity.

2. Materials and methods

2.1 Chemical and Reagent

Chemicals and reagent used for this research were of analytical grade.

2.2 Experimental animals

A total of 20 male Wistar rats weighing between 90-140 g were used for this study. The rats were acquired from the small Animal holding unit of the University of Ilorin. The animals were acclimatized for one week under room conditions with 12 hrs light/dark cycle. The

rats accessed normal rat feed and water ad libitum freely. The procedure for the handling of animal was approved by Landmark University Animal Care and Use Committee (LUAC - 0043B).

2.3 Collection and preparation of plant material

The African walnuts were purchased from the Oke-Onigbin market, Kwara State and authenticated at the Herbarium Unit, Department of Plant Biology, University of Ilorin, Nigeria. The voucher number for the specimen is UILH/001/1102.

The shelled nuts were rinsed with clean water and removed from their pods. The de-shelled nuts were washed and cut into pieces. After three days of fermentation by submerging of pieced nut in distilled water (1:2w/v), it was filtered, oven-dried at 60°C and mechanically ground to obtain fine particles. The processed walnuts and rat feed were manually mixed together, in two varying concentrations to give 5% and 10% walnut-supplemented feed respectively.

2.4 Determination of proximate composition

The standard guidelines as described by the Association of Official Analytical Chemists (AOAC, 2005) were followed for the determination of ash, crude fat and fibre, moisture content, carbohydrate and protein content of diets.

To determine the ash content, 20 g of each of the samples were placed in a furnace set at 550°C for 10 hrs until ash was obtained. The ash content was calculated by using this formula: % Ash = $(W3-W1)/(W2-W1) \times 100$ % where W1 = weight of oven-dried crucible, W2 = weight of crucible + sample before ashing, and W3 = weight of crucible + ash (AOAC, 2005).

Crude fat content was determined by employing the Soxhlet extraction technique (AOAC, 2005).

The crude fibre content was obtained by defatting 20 g of each sample with diethyl ether for 8 hrs and boiled under reflux for exactly 30 min with 200 mL of 1.25% H₂SO₄. It was filtered and later washed with boiling water to remove the acid. The residue was then boiled in a round bottom flask with 200 mL of 1.25% sodium hydroxide for another 30 mins and filtered into a previously weighed crucible. The crucible was then dried with samples in an oven at 100°C, left to cool in a desiccator and weighed. This was later incinerated in a muffle furnace at 600°C for 2 to 3 hrs and later allowed to cool in a desiccator and weighed (AOAC, 2005).

The moisture content was determined based on moisture evaporation. The aluminium dishes were dried

in the oven, cooled in desiccators and the weight of each dish was taken. A total of 5.0 g of samples were weighed into a sterile aluminium dish, weight of the dish and weight of the sample (in duplicate) were taken. This was transferred into an oven set at 80°C for 2 hrs and 105°C for 3 hrs respectively. This was removed and cooled in desiccators. Then the weight was measured using a measuring scale balance. It was transferred back into the oven for another one hour and then reweighed. The process continued until a constant weight was obtained. The difference in weight between the initial weight and the constant weight gained represents the moisture content (AOAC, 2005).

The carbohydrate value was calculated using this formula: Percentage of carbohydrates (%) = 100% - [moisture content (%) + Ash content + Crude fat content (%) + Crude protein content (%) + Ash content (%)].

The analysis of crude protein was carried out using the Kjeldahl method involving three steps (i.e. digestion, distillation and titration). The results of the protein content were expressed as percentage (%) value (AOAC, 2005).

2.5 Experimental protocol

Twenty rats were shared without a definite plan into four groups, each subjected to definite treatment as follows for six weeks:

- Group one received CdCl₂ (5 mg/kg body weight) and a normal diet without walnut,
- Group two received a normal diet only without walnut,
- Group three received CdCl₂ (5 mg/kg body weight) and 5% walnut supplemented diet and
- Group four received CdCl₂ (5 mg/kg body weight) and 10% walnut supplemented diet.

2.6 Collection and preparation of experimental samples

A whole day after exposure to the last dose and an overnight fast, the animals were sacrificed anaesthetically via light diethyl ether exposure. The liver and brain were excised and weighed. The blood samples were also collected into clean EDTA and heparin bottles for haematological and biochemical assays respectively. Sample of blood in heparin bottles was subsequently centrifuged for 10 mins at 5000×g to obtain plasma for further analysis.

The liver and brain were then homogenized in 0.1 M phosphate buffer (1:4 w/v) using a tissue homogenizer. The homogenates were centrifuged at 5000×g for 10 mins to obtain supernatant, which was collected with

micropipette into sample bottles. The liver, brain homogenates and plasma were stored frozen at -20°C until biochemical analyses were carried out.

2.7 Biochemical analyses

The biochemical indices were determined in the liver and brain homogenates, and plasma using UV/visible spectrophotometer (Jenway). The total protein, superoxide dismutase (SOD), reduced glutathione (GSH) and lipid peroxidation (MDA) were determined in both the liver and brain as described by Gornall *et al.* (1949) with slight modification, Misra and Fridovich (1972), Beutler *et al.* (1969) and Kei (1978) respectively. Liver Alkaline phosphatase (ALP) was determined as described by Wright *et al.* (1972). Also, brain acetylcholinesterase (AChE) was determined as described by Ellman *et al.* (1961). Radox assay kits (Crumlin, UK) were used to assess the lipid profile including cholesterol (TC) and triglyceride (TG) using the plasma.

2.8 Haematological indices

For the determination of white blood cells (WBC), red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular haemoglobin concentration (MCHC), mean cell volume (MCV), mean corpuscular haemoglobin (MCH) and platelet count (PLT), an automatic blood cell counter was used.

2.9 Statistical analysis

The results are expressed as mean ± standard error of mean (SEM). Statistical differences in the mean values were determined using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test on Graphpad Prism 5. Results with p<0.05 were considered significant.

3. Results

The walnut supplemented diet was assessed for proximate composition using the AOAC methods. There was no significant difference in the content of crude lipid and crude fibre (Table 1). Ash content was significantly different from the control (normal diet) in both 5 and 10 % respectively. The walnut supplemented diet contained increasing protein content in the 5 and 10 % respectively.

Cadmium exposure did not significantly change the rat's body, liver and brain weight as shown (Figure 1). Furthermore, while the liver-body weight ratio was not significantly altered, the brain-body weight ratio was significantly increased (p<0.05) in the group supplemented with 10 % walnut.

Administration of walnut supplemented feed did not

Table 1. Proximate composition (%) of walnut supplemented feed

	Crude fat	Moisture	Ash	Carbohydrate	Crude fibre	Crude protein
0%(CONTROL)	8.38±0.85	4.02±0.19	2.92±0.03	71.45±1.81	2.79±0.43	10.44±0.30
5%	7.64±0.56	4.67±0.00	13.27±0.75**	59.26±0.41*	3.58±0.04	11.59±0.18
10%	7.97±0.56	1.39±0.31**	10.87±0.52**	64.7±0.90	3.14±0.08	11.92±0.23*

Values are presented as mean±SEM, (n = 2). Statistical difference is relative to control, *P < 0.05 and ** P < 0.01.

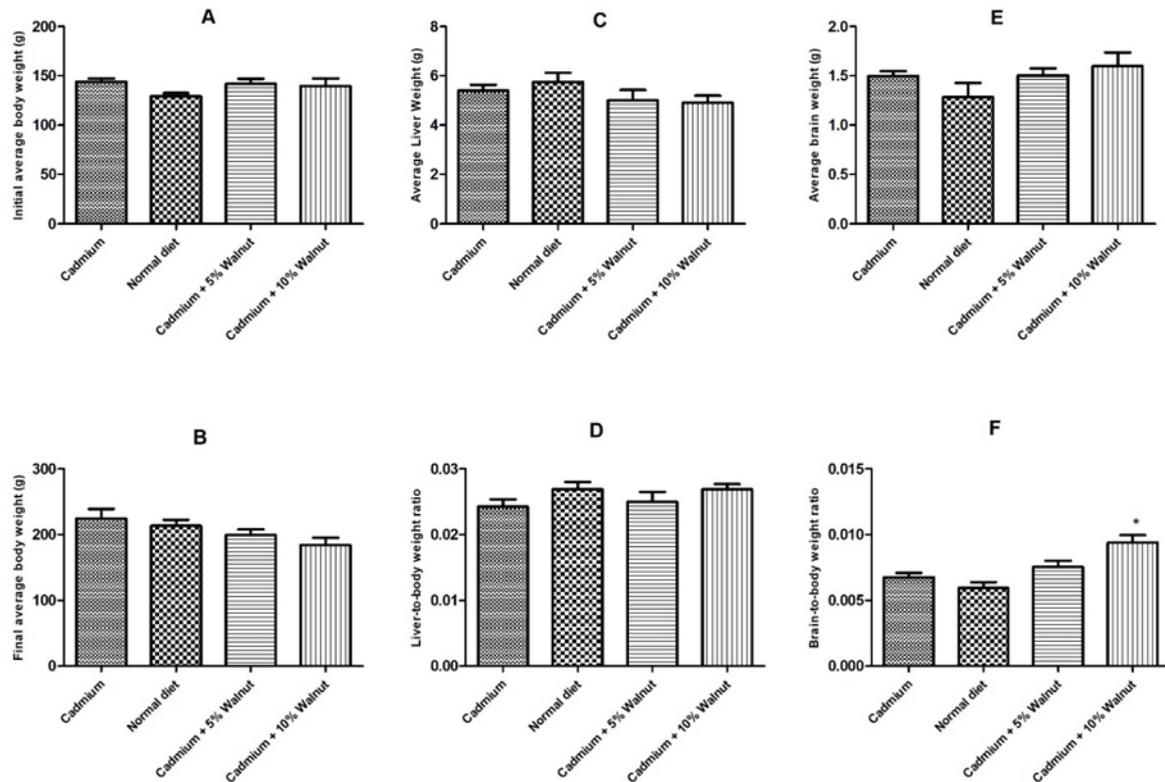


Figure 1. The effect of walnut supplemented diet. **A:** Initial rat weight; **B:** Final rat weight; **C:** Rat liver weight; **D:** Liver to body weight ratio; **E:** Rat brain weight; **F:** Liver to bodyweight ratio. Bars are mean ± SEM, n = 5. Statistical difference is relative to Cd, *P < 0.05.

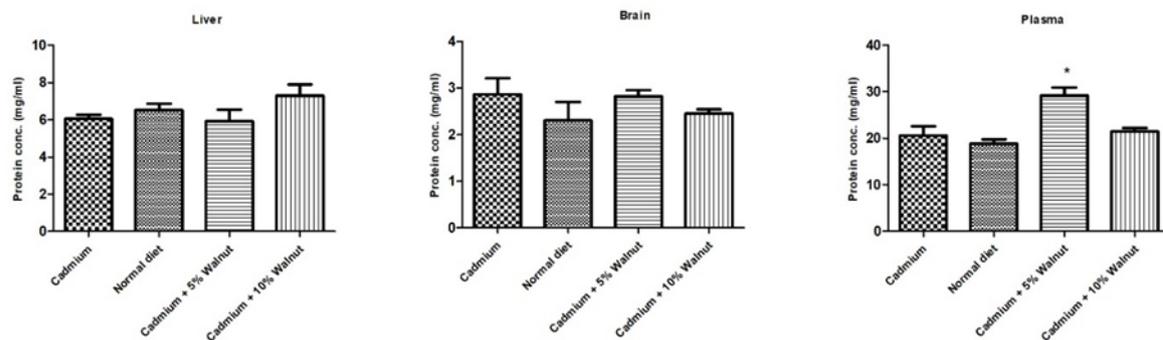


Figure 2. The effect of walnut supplemented diet on total protein concentration of rats administered with cadmium chloride. Bars are mean±SEM, n = 5. Statistical difference is relative to Cd, *P < 0.05.

significantly ($p > 0.05$) alter the protein content of rat liver and brain when compared with the Cd group. However, a significant increase ($p \leq 0.05$) in rat plasma protein was observed in the group fed 5% walnut supplemented feed when compared Cd only group (Figure 2).

Exposure to Cd increased the acetylcholinesterase activity in the brain (Figure 3). Walnut supplementation reversed the trend relative to the control. The activity of alkaline phosphatase was suppressed due to Cd exposure while the two group of supplemented diet produced

inconsistent responses.

The trend of the SOD and CAT were not consistent following supplementation with walnut to Cd intoxicated rats (Figure 4).

Exposure to Cd escalated SOD and CAT in both the liver and brain of rats. Supplementation with 5% and 10% walnut seemed to repress the antioxidant enzyme, especially in the liver.

Glutathione levels in the liver seem not to be

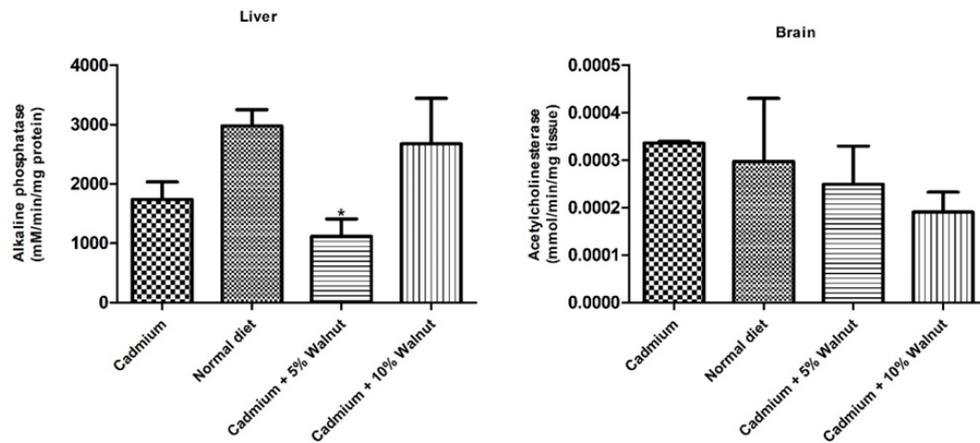


Figure 3. The effect of walnut supplemented diet on liver alkaline phosphatase and brain acetylcholinesterase activities of rats administered with cadmium chloride. Bars are mean \pm SEM, n = 5. Statistical difference is relative to Cd, *P < 0.05.

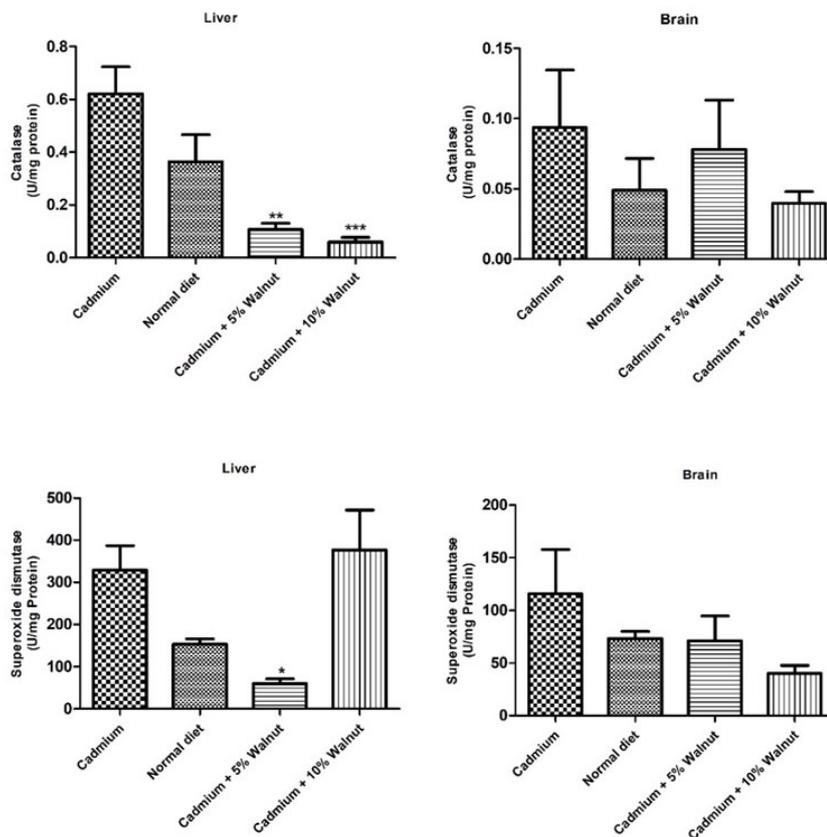


Figure 4. The effect of walnut supplemented diet on catalase and superoxide dismutase activity of rats administered with cadmium chloride. Bars are mean \pm SEM, n = 5. Statistical difference is relative to Cd, *P < 0.05, **P < 0.01 and ***P < 0.001.

Table 2. The effect of walnut supplemented feed on haematological parameters in rats administered with cadmium chloride

Group/Parameters	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	PLT
Cadmium	9.70 \pm 5.95	7.74 \pm 1.71	11.63 \pm 2.45	38.43 \pm 8.37	49.70 \pm 0.82	15.03 \pm 0.21	30.27 \pm 0.81	482.67 \pm 210.17
Normal diet	7.83 \pm 8.03	8.65 \pm 1.28	13.10 \pm 2.77	43.27 \pm 5.14	50.13 \pm 1.33	15.03 \pm 0.92	30.03 \pm 2.66	670.03 \pm 38.68
5% Walnut Feed	9.53 \pm 2.20	8.64 \pm 0.34	12.90 \pm 0.80 ^a	42.17 \pm 3.69	48.77 \pm 2.34	14.93 \pm 0.40	30.63 \pm 1.07	838.00 \pm 206.83
10% Walnut Feed	8.03 \pm 2.34	8.27 \pm 0.96	11.17 \pm 1.00	39.40 \pm 5.05	47.57 \pm 0.64	13.67 \pm 2.31	28.77 \pm 5.17	549.67 \pm 79.46

Values are presented as mean \pm SEM, n = 5.

significantly altered by supplementation with walnut across all the groups. However, the brain glutathione levels were significantly diminished following walnut supplementation in rats exposed to Cd. Malondialdehyde concentration in the brain increased significantly on Cd exposure but this was tangibly normalized in both groups

supplemented with walnut (Figure 5).

Exposure to Cd significantly (P<0.05) raised the cholesterol and triglyceride levels. Walnut supplemented diet upturned the Cd-induced perturbation in lipid profile (Figure 6).

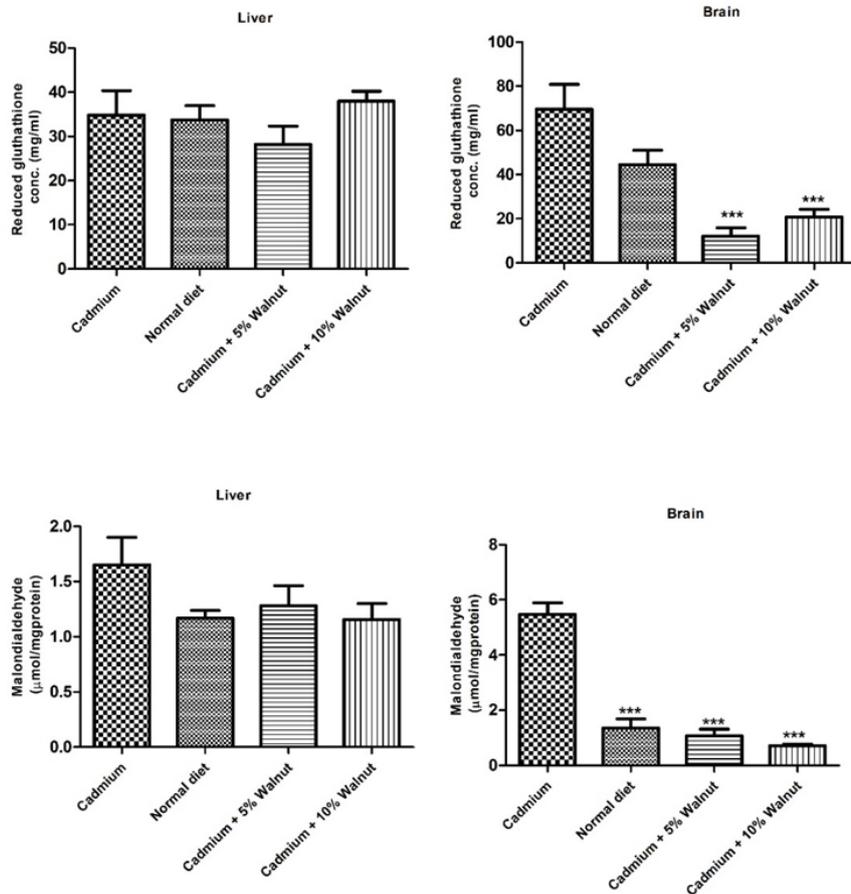


Figure 5. The effect of walnut supplemented diet on malondialdehyde and reduced glutathione level of rats administered cadmium chloride. Bars are mean±SEM, n = 5. Statistical difference is relative to Cd, ***P < 0.001.

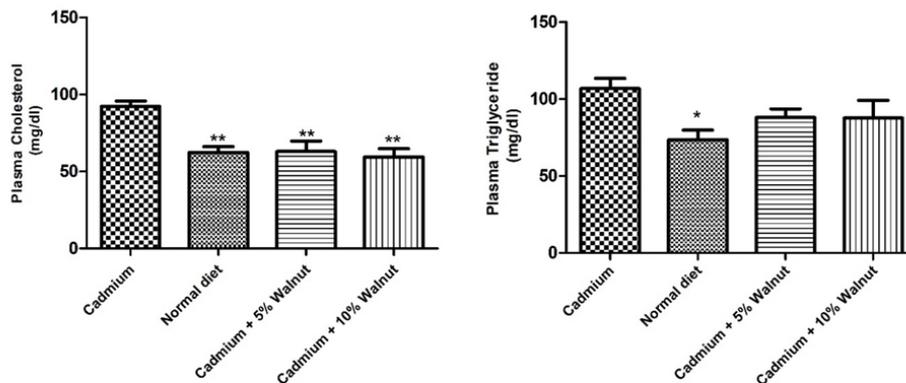


Figure 6. The effect of walnut supplemented diet on plasma cholesterol and triglyceride of rats administered with cadmium chloride only. Bars are mean±SEM, n = 5. Statistical difference is relative to Cd, *P < 0.05 and **P < 0.01.

There was no significant difference observed in the haematological parameters across both supplemented groups compared to Cd only group. The group given 10% walnut was statistically similar to the control group in the concentration of platelets (Table 2).

4. Discussion

Cadmium is one of the most paramount environmental toxicants and serves no known biological function. Exposure to Cd through consumption of contaminated food and industrial occupation poses a considerable public health threat and has the propensity to cause dysfunction/impairment in several organs including the liver, kidney, brain, lungs, testes and

ovaries (Claudio *et al.*, 2019).

This current study explored the protective possibility of fermented walnut supplemented diet against Cd-induced toxicity and oxidative stress in the liver and brain of male rats.

The proximate analysis of the supplemented diet showed slightly elevated levels of protein, ash and fibre content compared to the normal diet. This finding is in tandem with a previous study by Arinola and Adesin (2014) and may indicate the nutritional richness and health-enhancing benefits of walnut.

The current study revealed that levels of some blood parameters namely: RBC, haemoglobin, packed cell

volume (HCT) and platelets were lowered in the Cd intoxicated rats. This result is in concert with previous investigations (Nikolić *et al.*, 2015; Donmez *et al.*, 2019). While a variety of tissues/organs are biochemically distressed on exposure to Cd, Abadin *et al.* (2007) reported that the hematopoietic system and the blood represent a critical toxicity target of heavy metals such as Pb and Cd. These metals can bind to the RBC membrane and plasma albumin and is then conveyed to the liver (Andjelkovic, 2019). However, this effect was clearly reversed in groups fed walnut supplemented-feed thereby protecting against Cd toxicity.

Prior investigations revealed that exposure to chemical toxicants may bring about an alteration in the weight of the body or organ which may indicate toxicity (Zhai *et al.*, 2015; Nwonuma *et al.*, 2016; Adeyemi *et al.*, 2017). The exposure to Cd in this study brought about inconsistent alterations in the organ-to-body weight ratio in rat liver and brain. While the walnut supplemented diet in Cd exposed rats restored the weight of the liver compared to the group fed normal diet only, the supplemented feeds seem to have a hypertrophic effect on the brain which could possibly be indicative of neurotoxicity. This finding however is disagreeable with a report by Poulose *et al.* (2014) that polyphenolic compounds found in walnuts (*Juglan regia*) not only reduced the oxidant and inflammatory load on brain cells but also enhanced communication between neurons, increased the formation of neuron in the brain and facilitated sequestration of insoluble toxic protein aggregates.

In the present study, a non-significant increase in brain AChE activity was observed in the Cd exposed group relative to the control. Conclaves *et al.* (2012) similarly reported that dietary Cd exposure for five months raised AChE activity with concomitant impairment of cognitive function in rats. However, the walnut supplemented diet in this study seems to slightly reduce AChE activity. Some other researchers contrarily have reported a decrease in the AChE level in the brain following Cd intoxication (Abu-Taweel, 2016; Vijaya *et al.*, 2020).

Liver alkaline phosphatase activity declined in the Cd exposed group but the two grades of walnut supplemented diet produced dose-independent outcomes. Treviño *et al.* (2015) reported that chronic exposure of Cd produces an *in vivo* inhibition of ALP with kinetic studies suggesting that inhibition of ALP occurs by a replacement of Cd by Zn in the native enzyme.

The antioxidant enzymes such as SOD and CAT which provide first-line defence against reactive oxygen species (ROS) were evaluated. Living organism

normally produces ROS due to cellular metabolism. These perform vital physiological cell processes at low concentrations but at high concentrations, they could invoke cellular impairment to proteins, lipids and DNA (Birben *et al.*, 2012).

SOD catalyses the dismutation of superoxide radicals to hydrogen peroxide (H₂O₂) and subsequently degraded to generate one molecule of each of water (H₂O) and oxygen (O₂) by catalase (Gravato *et al.*, 2006). While the liver SOD activity increased significantly in the group administered Cd, there was a decrease in its activity in the brain relative to the control. Walnut supplementation remarkably enhanced the liver SOD activity of Cd exposed rat probably due to the rich polyphenols which have the potential to modulate oxidative stress. However, the brain SOD was significantly depressed by Cd, but these effects seem un-amended by the supplementation.

MDA is a major biomarker of oxidative stress and the most often studied product of lipid peroxidation. Cellular membrane lipids and proteins could become oxidatively impaired in the highly oxidized environment created as a result of elevated levels of free radicals and ROS (Khan *et al.*, 2015).

In this study, Cd exposure triggered an increase in MDA level and reduced glutathione in rat's liver and much more in the brain relative to the rats fed a normal diet. The high level of MDA in both the liver and brain of Cd exposed rats indicate possible oxidation in the lipid and protein which form the membrane components leading to the generation of ROS in these vital organs.

In a related study, Ogunrinola *et al.* (2015) reported a dose-dependent (100, 200 and 300 ppm Cd) increase of MDA level in the liver and brain of rat exposed to Cd relative to control. Meanwhile, MDA level was drastically abated in the walnut supplemented group exposed to Cd similarly to a normal diet, attesting to the radical scavenging potential of walnut.

Several epidemiological studies have reported that elevated concentration of plasma cholesterol due to dietary or occupational exposure to a toxicant is an important risk factor for cardiovascular disease (Ademuyiwa *et al.*, 2005). Cadmium has been reported to adversely affect lipid profile by way of inducing lipid peroxidation (Olisekodiaka *et al.*, 2012). Furthermore, exposure to Cd perturbed the lipid profile substantially increasing cholesterol and triglyceride levels in the rat exposed to Cd only, but again the inclusion of walnut in the rat diet reversed these alterations of lipid profile. These outcomes agree with previous findings which have established the perturbation of lipid profile by Cd

exposure in rats (Samarghandian *et al.*, 2015) and the lipid-lowering and cardioprotective effect of walnut (Banel and Hu, 2009; Bashan and Bakman, 2018). The modulatory effect of walnut diet improved blood parameters, ameliorated oxidative stress and restored lipid profile alteration necessitating its incorporation in the routine diet for improved wellness and health.

5. Conclusion

Conclusively, the walnut supplementation in this study appears to have abated the heavy metal toxicity due to the abundant ions/phytochemicals present in the nut. These results suggest that heavy metal intoxication could be managed variably by the nutritional benefits of walnut supplement in food.

Conflict of interest

The authors declare no conflict of interest.

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