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Effects of *Pterocarpus mildbraedii* Leaf Extract and Its Fractions on Cadmium and Lead Chloride-Induced Testicular Damage in Male Albino Rats

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***Abstract*—**Lead (Pb) and Cadmium (Cd) are toxic, non-essential transition metals that pose many health risks for both humans and animals. They are environmental toxicants which contribute to testicular damage resulting to infertility problem among male populace worldwide. Chelating agents used for lead and cadmium toxicity are not readily available, toxic, expensive and unable to mop up most of the toxic metals accumulated in various organs. In this study, the effect of crude extract (CE), ethyl acetate fraction (EF) and acetone fraction (AF) of *Pterocarpus mildbraedii* leaf extract was assessed on cadmium-lead chloride induced testicular damaged in male albino Wistar rats. CE of the leaf was obtained by extracting in absolute methanol which was further subjected to solvent partitioning via vacuum liquid chromatographic (VLC) techniques using ethyl acetate, acetone and 70% methanol. A preliminary phytochemical screening and *in vitro a*ntioxidants guided activities on the CE and fractions were determined using standard methods. EF, AF and CE which exhibited significant *in vitro* activity were subjected to an *in vivo* study using Wistar rats. *In vivo* antioxidant markers, male reproductive hormones, testicular enzymes and DNA damage markers were analyzed on the rats' testes supernatant. AF had the highest quantities of phenols (319.00 mg/g), flavonoids (8.87 mg/g) and tannins (8.87 mg/g) while methanol and EFs were richer in saponins (135.32 µg/g) and alkaloids (38.34 µg/g) respectively. A dose dependent 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric reducing antioxidant power (FRAP) and lipid peroxidation were observed in all the extract with high antioxidants power in CE and AF. Administration of lead-cadmium chloride solution significantly ($p > 0.05$) decreases the testicular superoxide dismutase (SOD) activity to 6.82 unit/mg protein, Catalase (CAT) activity to 8.07 of H_2O_2 consumed/unit/mg protein and Glutathione (GSH) concentration to 31.30 ug/mg protein. There was a concomitant increase in the level of Malondialdehyde (MDA) to a value of 23.70 mmol/mg protein. In addition, lead-cadmium chloride solution significantly ($p > 0.05$) increases the testicular marker enzymes (Alkaline phosphatase (119.57 u/L), lactate dehydrogenase (357.05 u/L), Acid phosphatase (98.65 u/L)) and DNA damage markers (conjugated dienes (93.39 nmol/mg protein), carbonyl protein (35.39 nmol/mg protein), DNA fragmentation percentage (32.12%)) with lowered testicular hormones (Testosterone (3.1 ng/mL), Follicle stimulating (0.35 IU/mL) and Luteinizing hormones (0.15 IU/mL)) of the animals in negative control group when compared with other treated groups. Treatment with *Pterocarpus mildbraedii* leaf extract reverts the observed changes with the best activities found in the CE and AFs in a dose dependent manner. *Pterocarpus mildbraedii* leaf extract ameliorated the lead/cadmium induced testicular damage in male albino rats. The restoration of the aforementioned parameters by some of the extract dosages were comparable to the standard drug with higher activities in the crude and AF. Therefore, *Pterocarpus mildbraedii* leaf extract can be explored further for the management of lead/cadmium induced toxicity.

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***Keywords*—**Cadmium, lead, P*terocarpus mildbraedii,* testicular damage.

I. Introduction

LEAD and cadmium are heavy metals that are toxic to the environment and humans. They are naturally distributed but increased greatly in the environment from their involvement in industrial activities. Industries connected with manufacturing of paints, batteries, pigments, pipes, ceramic, toys, ammunitions, cooking wares, smelting and mining, are well-known emitters of Cd and Pb [1]. Other sources are jewelry, some cosmetics and traditional medicines [2]. The risk of human exposure to these metals is on the high side due to their increased emission into the environment and their non-biodegradability. Lead and cadmium co-exist in the environment most times and are by products of many industrial activities. These metals enter and accumulate in different organs (kidney, liver, testicles, pancreas, thyroid, salivary glands, bone, brain and reproductive system) of the body in human and animal causing severe damage ranging from cellular degeneration, inflammation to cancers [3]. The main routes of Cd and Pb exposure into humans are via ingestion and inhalation due to their contamination in food, water, air, and tobacco leaves. The primary target organ for lead and cadmium toxicity is the male reproductive organ. Lead and cadmium are known to reduce male infertility by decreasing sperm density, total sperm number and viability with increase in the number of pathological spermatozoa, decreased libido, altered spermatogenesis, chromosomal damage and changes in serum testosterone [2]-[4] with marked negative effect in the overall testicular function [5], [6].

Male infertility caused by Pb and Cd toxicity has been reported to be associated with oxidative stress leading to an alteration in antioxidant defense system hence resulting in decrease in antioxidant enzymes and marked lipid peroxidation [6]. Pb and Cd toxicity has been suspected to be one of the main reasons of male infertility related with reduced testicular function and associated testicular oxidative stress in animals and human that reside in industrial areas where these two metals are used for different manufacturing processes [7], [8]. Oxidative stress causes damage to both mitochondrial and nuclear DNA and has adverse effects on the structural and functional integrity of sperm which is a major cause of defective

sperm function and male infertility [9].

Animals especially humans are exposed to mixtures of different heavy metals rather than an individual metal, thus, it is paramount to establish whether the former exerts a more noticeable effect compared to the latter. There are no effective ways to reduce the concentrations of Cd an Pb in food and as such man is constantly and consistently exposed to the intake of these metals thus plants rich in antioxidants capable of chelating these metals and increasing the oxidative capacity (which decreases oxidative damage to internal organs) are sought. Many plants are known to contain phyto-antioxidants such as phenols, flavonoids, terpenes, alkaloids and tannins which are capable of reversing oxidative damage resulting from oxidative stress [10], [11].

Pterocarpus mildbraedii belong to the family Papilionaceae which is commonly called paduk. It has different local names in Nigeria "Oha" in Igbo, "Madobiyar rafi" in Hausa, "Urube" in Edo, "Geneghar" in Ijaw and "Kakupupu" in Urhobo [12]. It is found majorly in some West African countries like Nigeria, Ghana, Liberia, Cameroun, and Cote d'Ivoire. It leaves are commonly consumed as vegetable and for different medicinal purposes in Nigeria. They are valued for their high vitamin content and minerals as well as phytochemicals such as phenols, flavonoids, tannins and alkaloids [13]. The protective effect of the leave of *P. mildbraedii* against oxidative stress induced liver damage and production of active principles with relevant anti-inflammatory potential has been reported [12], [14]. In the search for a plant that is readily available with less side effects and with high abundance of antioxidant to combat the toxicity of the coexistence of Pb and Cd, this study was therefore designed to evaluate the effects of *P. mildbraedii* leaf extract and its fractions on cadmium and lead chloride-induced testicular damage in male albino rats.

II. Materials and Methods

A. Collection and Identification of Plant

Fresh leaves of *Pterocarpus mildbraedii* were collected from a farm in Anambra State, Nigeria and identified by Dr. Ugbabe, G. of National Institute of Pharmaceutical Research, Abuja, Nigeria (NIPRD). The leaf was assigned a Voucher/Specimen number of NIPRD/H6713 and deposited at NIPRD herbarium.

B. Chemicals and Reagents

The chemicals and reagents used in this study were of analytical grade. Cadmium chloride (Merck, Germany) and lead chloride (Centrohem, Serbia) were used for oral administration. Other chemicals and reagents used include methanol, acetone, ethyl acetate, 2, 2, diphenyl -1-picrylhydrayl (DPPH) radical, potassium hexacyanoferrate (III), ferric chloride, thiobarbituric acid (TBA), sodium dodecyl sulphate (SDS), ferrous sulphate, trichloroacetic acid (TCA). Lactate dehydrogenase, Acid Phosphatase (ACP) Randox and Alkaline Phosphatase (ALP) Agape assay kits were used. All other analytical chemicals and reagents for the estimation of antioxidant status were procured from Sigma-Aldrich Chemie (Germany).

C. Experimental Animals

Male Wistar rats (120.00 ± 5.20g) were obtained from the animal facility of Federal University of Technology, Minna (FUT MINNA), Niger State, Nigeria. The animals were housed in polypropylene cages under a controlled environment with 12 hours light/dark cycles, temperature of 28 ± 3 ℃ and relative humidity of 45-50%. The animals were fed on pelleted diet (Vital Feeds, Jos Nigeria) with supply of water *ad libitum*. The experiment was conducted according to protocol review of Canadian Council on Animal Care and use guideline [15].

D. Sample Preparation

The collected fresh leaves were rinsed with distilled water and air-dried at room temperature for a period of two weeks. After the leaves had dried, they were pulverized with blender (Binatone BLG 450, United Kingdom). The powder sample was kept in air-tight container until use.

E. Extraction and Fractionation of Pterocarpus mildbraedii Leaves Using VLC Techniques

200 g of the powdered sample was extracted with 1000 mL of methanol by cold maceration for 72 hours. Thereafter, the extract was filtered using Whatmann No. 1 filter paper and the extract was concentrated by air drying. The CE was mixed with small quantity of silica gel of 230-240 mesh with little quantity of methanol. The mixture was allowed to dry and spread on a sizeable filter paper placed on silica gel in a VLC column connected to a vacuum rotor. Appropriate volume of ethyl acetate, methanol and acetone were used for extraction sequentially. Each solvent was evaporated under reduced pressure in a rotary vacuum evaporator and the filtrate was lyophilized to obtain each fraction. The extracts were stored at the refrigerated condition at -4 °C.

F. Quantitative Phytochemical Constituents Determination

1. Total Phenol Determination

Briefly, in 10 mL of distilled water, 0.01 g of each extract was dissolved, then oxidized and neutralized by adding 2.5 mL of 10% Folin-Ciocalteu's reagent and 2 mL of 7.5% sodium carbonate respectively. The reaction mixture was allowed to stand at 45 ◦C for 40 minutes. Absorbances were read at 765 nm using double beam Shimadzu UV spectrophotometer, UV-1800. Gallic acid was used to prepare the calibration curve [15]

2. Flavonoid Determination

In this method, in 10 mL of distilled water 0.01 g of each extract was weighed and dissolved. Afterwards, to a test tube containing 1.5 mL of absolute methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate, 0.5 mL of each extract was added and 2.8 mL of distilled water and allowed to stand at ambient temperature for 30 minutes. The absorbances were read at 415 nm with double beam Shimadzu UV-spectrophotometer, UV-1800. Quercetin was used to prepare the calibration curve [16].

3. Alkaloid Determination

Alkaloid content of the extracts was determined as described by Oloyede [17]. Briefly, 0.5 g of each of the extract were

weighed and dissolved in 5 mL of mixture of 96 % ethanol : 20 % H_2SO_4 (1:1) and then filtered using Whatman No 1 filter paper. 1 mL of each filtrate was then added to a test tube containing 5 mL of 60% H_2SO_4 and allowed to stand for 5 minutes. Thereafter, 5 mL of 0.5% formalin was added and allowed to stand at room temperature for 3 hours. The absorbance was read at wavelength of 565 nm. Vincristine extinction coefficient (E_{296}, ethanol {ETOH}= 15136 $M^{-1}cm^{-1}$) will be used as reference alkaloid.

4. Determination of Tannins

Briefly, 0.2 g of each of the extract were weighed into a 50 mL beaker and 20 mL of 50% methanol was added to it and covered with parafilm and heated in water bath at 80 °C for a period of 1 hour. The reaction mixtures were shaken thoroughly to ensure uniformity. Each extract was then filtered into separate 100 mL volumetric flasks, and 20 mL of distilled water, 2.5 mL of Folin-Denis' reagent, and 10 mL of sodium carbonate was added and mixed properly. The reaction mixtures were then allowed to stand for 20 minutes at room temperature for the development of bluish-green coloration. The absorbance was recorded at 760 nm using double beam Shimadzu UV-spectrophotometer, UV-1800. Standard tannic acid was used to prepare the calibration curve [18].

5. Determination of Saponins

Exactly 0.5 g of each of the extract were weighed and dissolved in 20 mL of 1 N HCl and boiled in water bath at 80 °C for 4 hours. The reaction mixtures were cooled and filtered. 50 mL of petroleum ether was added and the ether layer were collected and evaporated to dryness. Thereafter, 5 mL of acetone-ethanol (1:1), 6 mL of ferrous sulphate and 2 mL of concentrated sulphuric acid were added and allowed to stand for 10 minutes. The absorbances were taken at 490 nm. Standard saponins was used to prepare the calibration curve [17]

G. In vitro Antioxidant Activity Determination

1. DPPH Radical Scavenging Assay

The DPPH radical scavenging method was used to evaluate the free radical scavenging ability of the plant extracts as described by Oyaizu [19]. Different concentrations of extracts and ascorbic acid (50, 100, 200 and 400 µg/mL) were prepared from stock solutions (1000 µg/mL). Thereafter, to 1 mL of various concentrations of the plant extracts and ascorbic acid respectively, 2 mL of 0.004% DPPH in methanol was added. The reaction mixture was allowed to stand at 25 °C for 30 minutes. The absorbance of each test mixture was read in triplicates against blank at 517 nm using spectrophotometer (double beam Shimadzu UV-1800 series).

The percentage (%) antioxidant activity was calculated using the formula below:

$$\% = \frac{(\text{Absorbance of Blank} - \text{Absorbance of Test sample})}{\text{Absorbance of Blank}} \times 100$$

2. FRAP Assay

Different concentrations of 50, 100, 200 and 400 µg/mL of plant extracts and ascorbic acid (control) were prepared from stock solutions (1000 µg/mL). 1 mL of 0.2 M sodium phosphate buffer and that of 1% potassium hexacyanoferrate (III) were mixed with 1 mL of each plant extracts and ascorbic acid concentration. The reaction mixture was allowed to stand at 50 °C for 20 minutes. Thereafter, 1 mL of 10% TCA was added to the mixture and centrifuged at 3000 rpm for 10 minutes at room temperature. 1 mL of each supernatant obtained was mixed with 1 mL of distilled water and then 0.2 mL of 0.1% ferric chloride. The blank was prepared in the same manner as samples except that the extracts were replaced by distilled water. The absorbance of the test mixtures will be read at 700 nm [19]. The percentage antioxidant activity was calculated using the formula:

$$\% \text{ activity} = \frac{(\text{Absorbance of Blank} - \text{Absorbance of Test sample})}{\text{Absorbance of Blank}} \times 100$$

3. Inhibition of Lipid Peroxidation

Thiobarbituric acid reactive substances (TBARS) assay with mild modification was used to evaluate the inhibitory effects of the extracts on lipid peroxidation. Briefly, to 0.1 mL of extract or ascorbic acid (control) at various concentrations (50, 100, 200 and 400 µg/mL), 0.5 mL of 10% egg homogenate was added and made up to 1 mL with distilled water. Afterwards, 0.05 mL of $FeSO_4$ was added to the mixture and allowed to stand for 30 minutes. Then, 1.5 mL of acetic acid and TBA in SDS was added, respectively. The resulting reaction mixture was vortexed and allowed to stand at 95 °C for 1 hour. The reaction mixtures were cooled and 5 mL of butanol was added to each of the reaction mixtures and centrifuged at 1200 x g for 10 minutes and the absorbance of the upper organic layer was read at 532 nm [20].

The percentage inhibition of lipid peroxidation was determined using the formula:

$$\frac{(\text{Absorbance of Blank} - \text{Absorbance of Test sample})}{\text{Absorbance of Blank}} \times 100$$

H. Experimental Design and Procedures

A total of 45 rats were divided into 9 groups of five rats each. Cadmium and lead chloride were used in inducing toxicity in the rats at the dose of 10 and 5 mg/kg bodyweight respectively. The CE and fractions of the *Pterocarpus mildbraedii* were administered after 1 hour daily following oral administration of solution of cadmium/lead chloride for 2 weeks as follows;

- Group I: received normal saline (0.5 mL/kg bodyweight)
- Group 2: Cadmium/lead chloride + calcium disodium Edetate (10 mg/kg bodyweight)
- Group 3: Cadmium/lead chloride only
- Group 4: Cadmium/lead chloride + 200 mg/kg bodyweight CE
- Group 5: Cadmium/lead chloride + 400 mg/kg bodyweight CE
- Group 6: Cadmium/lead chloride + 200 mg/kg bodyweight AF
- Group 7: Cadmium/lead chloride + 400 mg/kg bodyweight AF
- Group 8: Cadmium/lead chloride + 200 mg/kg bodyweight

EF

- Group 9: Cadmium/lead chloride + 400 mg/kg bodyweight EF

On the 15th day of the experiment, animals were euthanized by cervical dislocation and blood samples were collected into serum bottles. These samples were allowed to clot at room temperature and sera were obtained by centrifuging at 3000 rpm for 10 minutes.

The testes of the experimental rats were collected and rinsed in 1.15% potassium chloride (KCl) and collected into already labelled sample bottles in ice. They were then weighed and homogenized with 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuge at 12,000 rpm for 15 minutes using cold centrifuge machine. The supernatant was then collected into properly labelled bottles and used for biochemical assays.

I. Determination of in vivo Antioxidant Markers

1. SOD Activity

SOD activity was determined using the method of [21]. The samples (1 mL) were diluted in 9 mL of distilled water to make 1 in 10 dilutions. An aliquot of the diluted samples was then added to 2.5 mL of 0.05 M carbonic acid buffer, pH 10.2 to equilibrate in the spectrophotometer and the reaction was initiated by the addition of 0.3 mL of freshly prepared 0.3 mM adrenaline to the mixture, which was quickly mixed by inversion. The reference cuvette contained 2.5 mL 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 seconds for 150 seconds. Calculations:

$$\text{Increase in absorbance per minute} = A_3\text{-}A_0/3.0$$

where A_0 = absorbance at 0 second, A_3 = absorbance at 150 seconds

$$\%\ \text{Inhibition} = \frac{\text{Increase in absorbance for sample}}{\text{Increase in absorbance for blank}}\ x\ 100$$

2. CAT Activity

The decomposition rate of hydrogen peroxide (H_2O_2) into O_2 and H_2O by the samples in 3 min were tested at 240 nm wavelength using a UV spectrophotometer (Shimadzu UV-Visible-1800 series, spectrophotometer). The reaction was initiated by addition of 20 µL of homogenate to the total 1 ml reaction mixture containing 100 µL of 100 mM H_2O_2 and 880 µL of 50 mM potassium phosphate buffer (pH 7.0). The change in the absorbance at 240 nm was measured at 20 s interval until 3 min. The enzyme activity was calculated by 0.0436 mM extinction coefficient of H_2O_2 at 240 nm. The CAT activities units/ml of seminal plasma were calculated based on the conversion ability of number of µmol/min H_2O_2 at 25 °C [22]. Calculations:

$$\text{CAT (U/mL)} = \frac{(\text{Change in Abs (sample)} - \text{Change in Abs (blank)})}{0.0436\ x\ volume\ of\ sample}$$

0.0436 = millimolar extinction coefficient of H_2O_2 at 240 m.

3. Reduced GSH Estimation

An aliquot of 100 µL of each of the samples was precipitated with 200 µL of 20% TCA and centrifuged at 2000 rpm at 4 °C for 5 minutes. Thereafter, 50 µL of the supernatant was mixed with 100 µL and 50 µL of 1 M phosphate buffer and 5 mM DNTB, respectively. The reaction mixture was incubated at 37 °C for 10 minutes, and the absorbance was read at 412 nm. The concentration of GSH was calculated from calibration curve [23].

4. Estimation of Malonaldehyde (MDA) Level

Briefly, 200 µL of the samples each combined with 0.2 mL of 8.1% SDS, 1.5 mL acetic acid and 1.5 mL TBA the solution made up to 4 mL with distilled water; then the solution was boiled for 60 minutes in a boiling water bath (95 °C); after cooling, the reaction product (TBA–MDA complex) was extracted by adding 1 mL of n-butanol-pyrimidine (15:1 v/v). The flocculent precipitate was removed by centrifugation at 3500 rpm for 15 min; then supernatant was obtained and absorbance reading of the supernatant at 532 nm against a blank that contains the reagents minus the samples. The MDA concentration of the sample was calculated using the adduct extinction coefficient of 1.56 x 105 M^{-1} cm^{-1} for MDA [24].

- MDA concentration (M) = $\frac{\text{Abs } 532}{155}$
- MDA conc. (µM): M x 1000 = MDA conc. (µM/mL) of the sample

J. Estimation of Testicular Function Marker Enzymes

ALP activity was measured using commercially available Agape ALP assay kit (Crumlin, UK) while Lactate dehydrogenase (LDH) activity and ACP activity were estimated using Randox kits (USA).

K. Determination of Hormone Profile

Assessment of total testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) was conducted using AccuBind and Diablab commercial ELISA kits

L. DNA Damage Markers

1. Conjugated Dienes

Briefly, testicular suspension was mixed with 5 mL, 2:1 (v/v) chloroform/methanol, and centrifuged at 1000 g for 10 min at 4 °C, to separate the aqueous layer from the organic layer. The chloroform layer containing the lipids was separated from the aqueous layer and evaporated to dryness at 45 °C. The lipid residue was re-suspended in 1.5 mL cyclohexane and the absorbance read at 233 nm against a blank containing only cyclohexane using UV/VIS Shimadzu spectrophotometer (UV-1800 series). Conjugated dienes were estimated using 2.52 x 10^4 extinction coefficient [25].

2. Fragmented DNA

The quantity of fragmented DNA in the testes was determined using the procedure described by [26]. Briefly, hepatocytes were centrifuged at 15 000 g, for 15 min at 4 °C. The supernatant was separated from the pellet and treated with TCA (1.50 mL, 10%). The pellet was also treated with TCA

(0.65 mL, 5%). The reaction mixtures were allowed to precipitate overnight in a refrigerator (4 ºC), centrifuged at 2500 g for 10 min. The reaction mixtures were boiled at 100 ºC for 15 min, cooled to room temperature and centrifuged at 2500 g for 5 min. The supernatants (0.5 mL) were treated with diphenylamine reagent (1 mL) and incubated at 37 ºC for 4 h. The absorbance was read at 600 nm using UV/VIS Shimadzu spectrophotometer (UV-1800 series). The fragmented DNA was calculated using expression:

$$\text{Fragmented DNA (\%)} = \frac{\text{(Absorbance of supernatant}}{\text{Absorbance of supernatant x Absorbance of pellet}}$$

3. Protein Carbonyl

Protein carbonyl concentration was determined according to the procedure described by Levine et al. [27]. Briefly, each of the testicular homogenates (100 mL) was mixed with an equal volume of 10 mM 2,4 dinitrophenylhydrazine (DNPH) prepared in 2.5 M HCl, and incubated in the dark for 1 h at room temperature, with intermittent shaking at every 15 min. After 1 h of incubation, TCA (10% w/v) was added and centrifuged at 3500 g for 20 min. The pellet was washed three times with ethanol/ethyl acetate (1:1 v/v) to remove unreacted DNPH and lipid remnants. The pellet was dissolved in 2 mL of 6 M guanidine hydrochloride and incubated at 37 ºC for 10 min, centrifuged at 1000 g for 10 min and the carbonyl content was determined by reading the absorbance of the samples at 370 nm using UV/VIS Shimadzu spectrophotometer (UV-1800 series). The carbonyl content (Mmol/mg protein) was estimated using an absorption coefficient of 22 000 M^{-1} cm^{-1}. Blank was treated the same way except that DNPH was replaced with 2.5 M HCl [27].

M.Statistical Analysis

Statistical analysis of the collected data was performed using SPSS Statistics 22. One One-way ANOVA followed by Duncan multiple comparison test was used to compare the means. Data are presented as mean ± standard error of the mean (SEM).

III. Result

A. Quantitative Phytoconstituents of Crude and Fraction of Pterocarpus mildbraedii Leaves Extract

Table I shows the phytochemical composition of the CE and fractions of *Pterocarpus mildbraedii* leaves. The phytochemicals are present in varying amounts in the CE and its fraction with phenols being the highest in AF

TABLE I
QUANTITATIVE PHYTOCONSTITUENTS OF CRUDE AND FRACTIONS OF *PTEROCARPUS MILDBRAEDII* LEAVES EXTRACT

	Phenols (mg/g)	Flavonoids (mg/g)	Tannins (mg/g)	Saponins (µg/g)	Alkaloids (µg/g)
CE	247.73 ± 0.61^{c}	4.63 ± 0.29^{a}	127.43 ± 0.76^{b}	129.45 ± 0.43^{c}	16.89 ± 0.38^{a}
Acetone	319.00 ± 0.38^{d}	8.87 ± 0.37^{c}	211.50 ± 0.31^{d}	120.24 ± 0.03^{b}	32.30 ± 0.06^{c}
70% methanol	287.65 ± 0.24^{b}	4.63 ± 0.32^{a}	109.30 ± 0.37^{a}	135.32 ± 0.26^{d}	24.22 ± 0.20^{b}
Ethyl acetate	218.00 ± 0.22^{a}	7.59 ± 0.22^{b}	151.10 ± 0.33^{c}	112.33 ± 0.25^{a}	38.34 ± 0.11^{d}

Values are expressed as mean ± standard error mean of triplicate determination. Values with same superscript on the same column are not significantly different at $p > 0.05$.

B. In vitro Antioxidant Activity of Crude and Fractions of Pterocarpus mildbraedii Leaves Extract

The results of the DPPH scavenging activity, FRAP and inhibition of lipid peroxidation by the crude and fractions of *Pterocarpus mildbraedii* leaf extract are presented in Figs. 1-3.

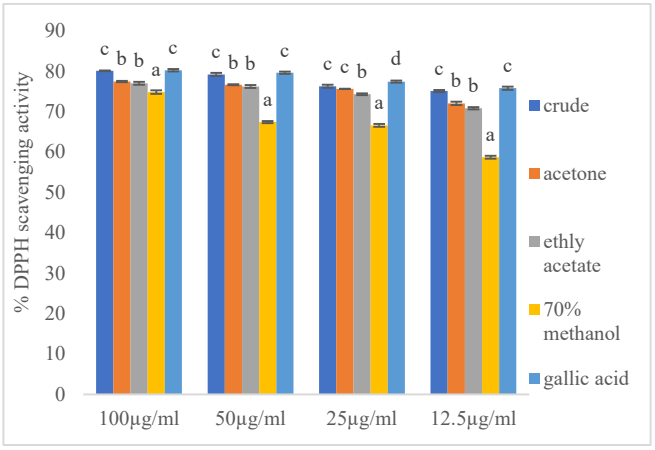

Fig. 1 DPPH Radical Scavenging Activity of *Pterocarpus mildbraedii* Leaves

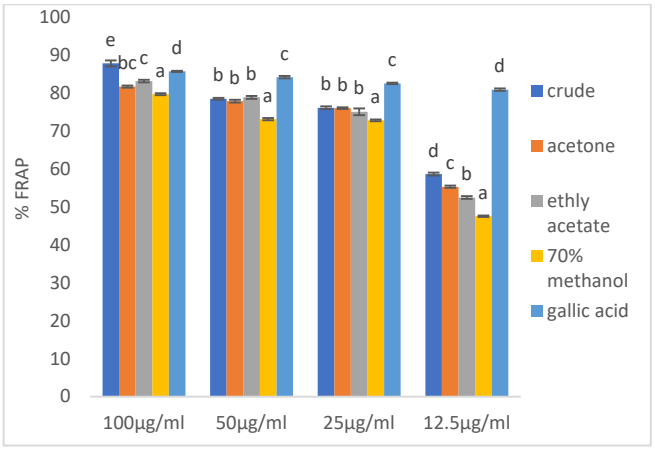

Fig. 2 FRAP of *Pterocarpus mildbraedii* Leaves

C. In vivo Antioxidant Activity of Crude and Fractions of Pterocarpus mildbraedii leaf Extract on Cadmium and Lead Chloride Induced Toxicity in Male Albino Rats

Figs. 4-7 show the results of SOD, CAT activities, reduced GSH concentration and level of MDA of crude and fractions of *Pterocarpus mildbraedii* leaf extract on cadmium and lead chloride induced toxicity in male albino rats.

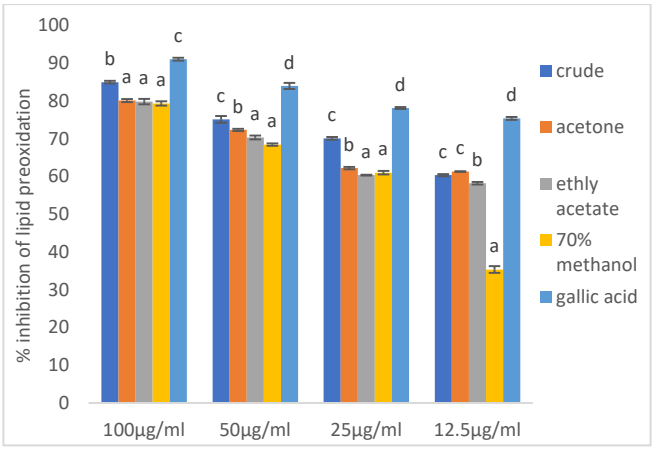

Fig. 3 Percentage Inhibition of Lipid Peroxidation of *Pterocarpus mildbraedii* Leaves

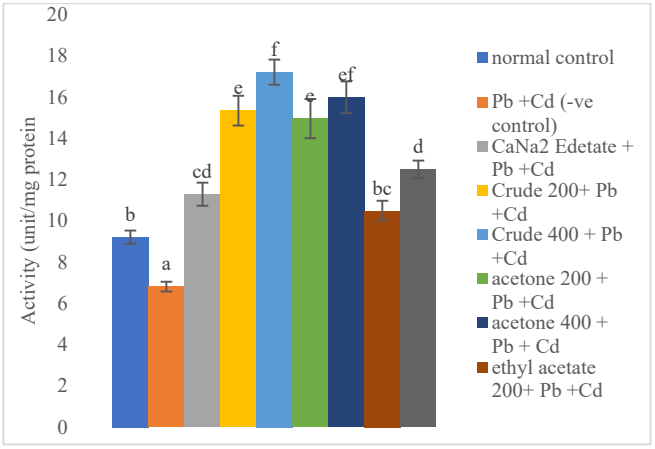

Fig. 4 Effect of CE and Fractions of *Pterocarpus mildbraedii* Leaf on SOD Activity in Cadmium and Lead Chloride Induced Toxicity in Male Albino Rats

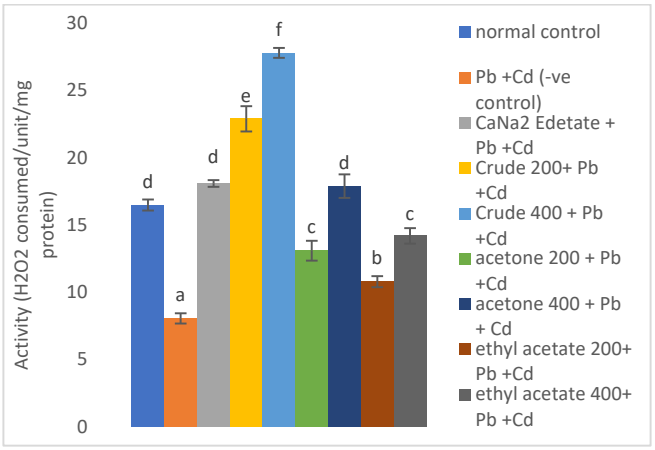

Fig. 5 Effect of CE and Fractions of *Pterocarpus mildbraedii* Leaf on CAT Activity in Cadmium and Lead Chloride Induced Toxicity in Male Albino Rats

D. Testicular Function Marker Enzymes

The results showing the effect of CE and fractions of *Pterocarpus mildbraedii* leaf on testes ALP, LDH and ACP activities in cadmium and lead chloride induced toxicity in male albino rats are depicted in Figs. 8-10.

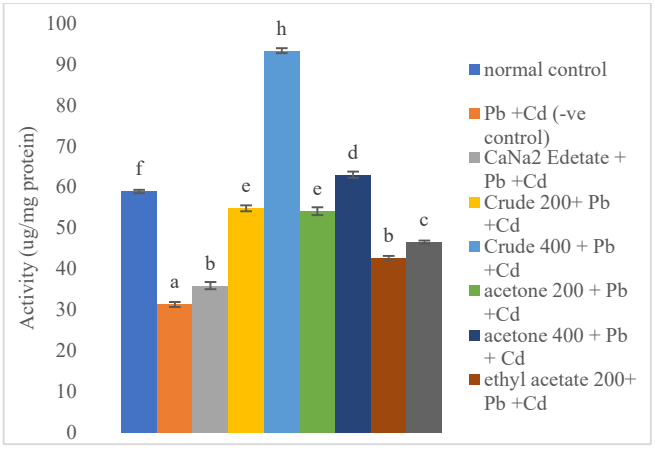

Fig. 6 Effect of CE and Fractions of *Pterocarpus mildbraedii* Leaf on GSH Activity in Cadmium and Lead Chloride Induced Toxicity in Male Albino Rats

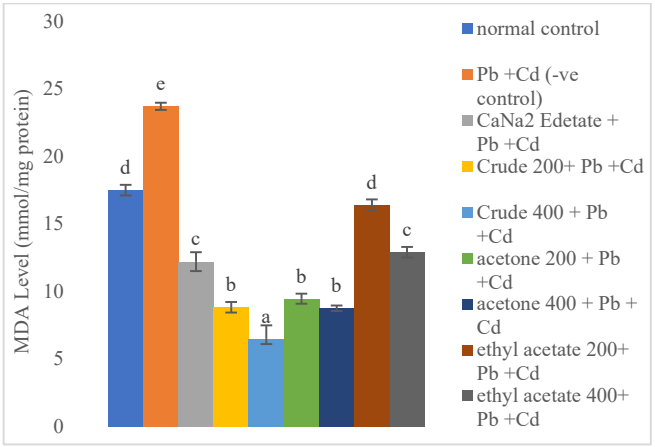

Fig. 7 Effect of CE and Fractions of *Pterocarpus mildbraedii* Leaf on MDA Level

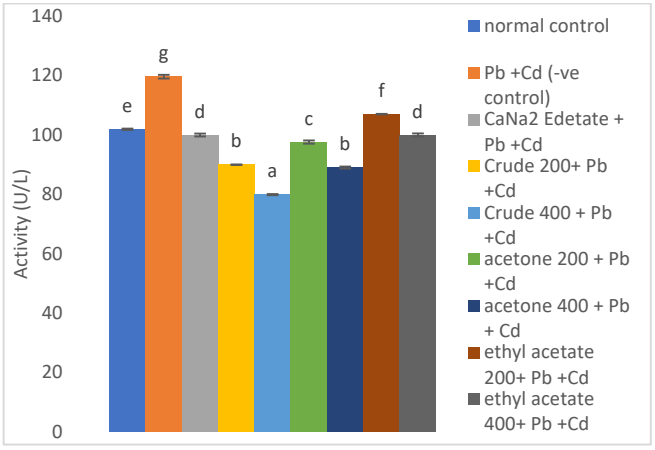

Fig. 8 Effect of CE and Fractions of *Pterocarpus mildbraedii* Leaf on Testes ALP Activity in Cadmium and Lead Chloride Induced Toxicity in Male Albino Rats

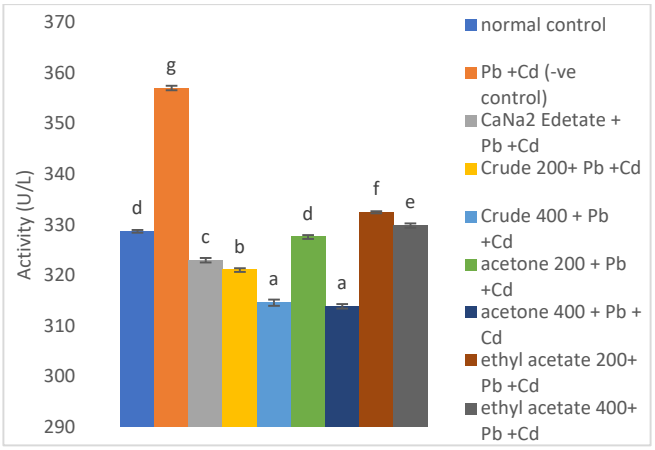

Fig. 9 Effect of CE and Fractions of *Pterocarpus mildbraedii* Leaf on Testes LDH Activity in Cadmium and Lead Chloride Induced Toxicity in Male Albino Rats

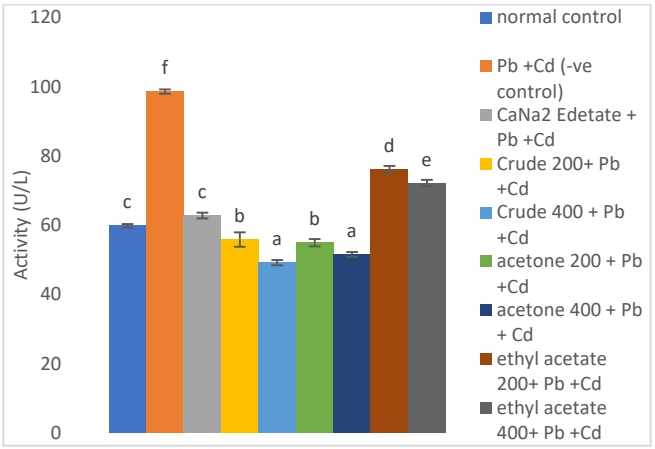

Fig. 10 Effect of CE and Fractions of *Pterocarpus mildbraedii* Leaf on Testes ACP Activity in Cadmium and Lead Chloride Induced Toxicity in Male Albino Rats

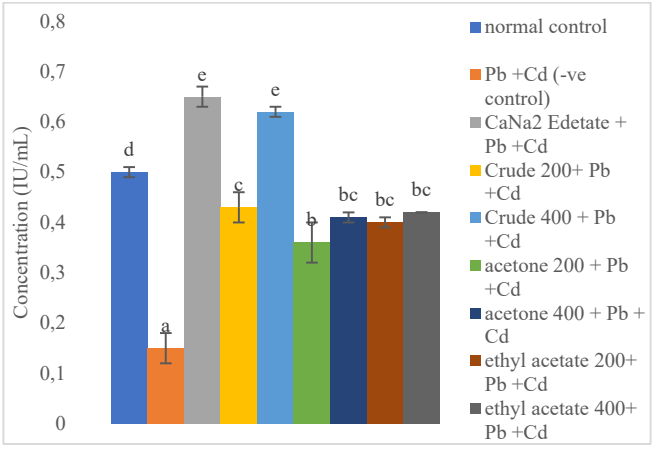

Fig. 11 Effect of CE and Fractions of *Pterocarpus mildbraedii* Leaf on FSH Activity in Cadmium and Lead Chloride Induced Toxicity in Male Albino Rats

E. Hormonal Profile

The hormonal profile (FSH, Testosterone and LH) of male albino rats induced with cadmium and lead are shown in Figs. 11-13.

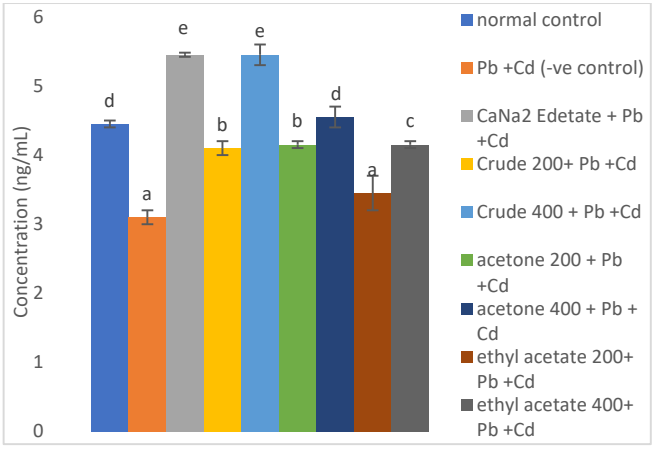

Fig. 12 Effect of CE and Fractions of *Pterocarpus mildbraedii* Leaf on Testosterone Activity in Cadmium and Lead Chloride Induced Toxicity in Male Albino Rats

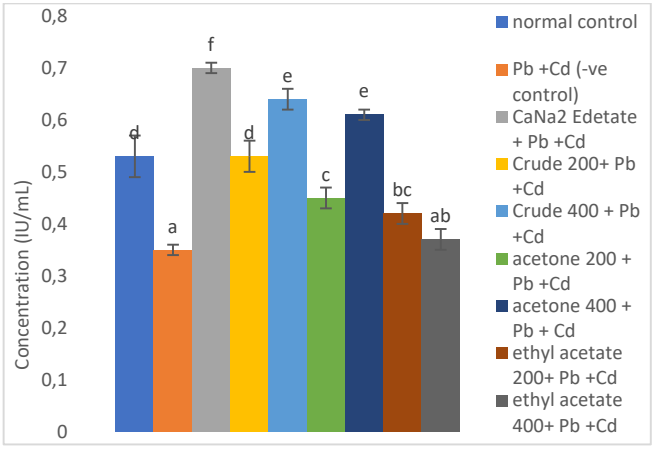

Fig. 13 Effect of CE and Fractions of *Pterocarpus mildbraedii* Leaf on LH Activity in Cadmium and Lead Chloride Induced Toxicity in Male Albino Rats

F. Effect of CE and Fractions of Pterocarpus mildbraedii Leaf on Markers of DNA Damage

The effect of CE and fractions of *Pterocarpus mildbraedii* leaf on markers of DNA damage are presented in Figs. 14-16.

IV. Discussion of Results

The quantitative phytochemical analysis showed the presence of secondary metabolites such as phenols, flavonoids, tannins, saponins and alkaloids in all of the fractions as well as the CEs in varying quantities. These findings conceded with that of Usunobun and Igwe, who also reported the presence of these secondary metabolites in the leaves of *Pterocarpus mildbraedii* [28]. Phenolic compounds such as phenols, flavonoids and tannins have been shown to possess antioxidant, antidiabetic, anti-inflammatory and antimicrobial activities, among others [29]-[31]. Alkaloids have been reported to possess pharmacological activities such as antimalarial,

antiasthma, anticancer, vasodilatory, antiarrhythmic and analgesic activities, while saponins have been associated with serum cholesterol lowering, immune-modulatory and antitumor activities [32], [33]. Thence, the pharmacological activities exhibited by the fractions as well as the CE could be traceable to their phyto-constituents.

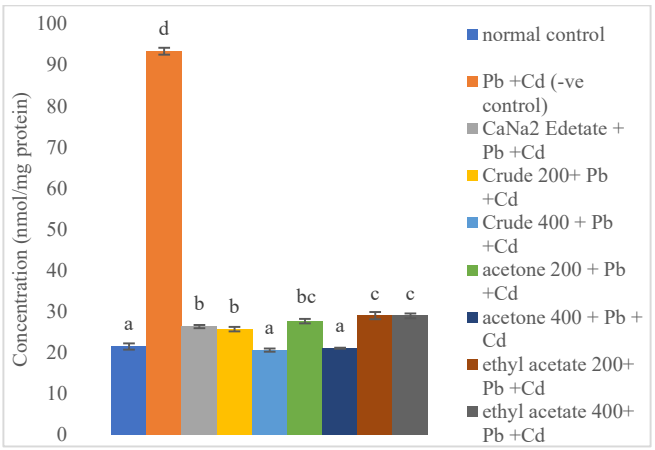

Fig. 14 Effect of CE and Fractions of *Pterocarpus mildbraedii* Leaf on Testes conjugated dienes in Cadmium and Lead Chloride Induced Toxicity in Male Albino Rats

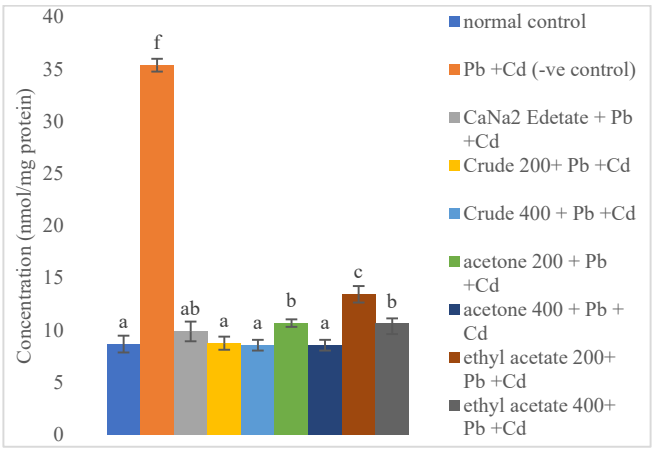

Fig. 15 Effect of CE and Fractions of *Pterocarpus mildbraedii* Leaf on Testes protein carbonyl in Cadmium and Lead Chloride Induced Toxicity in Male Albino Rats

DPPH is a stable free radical widely employed in the assessment of free-radical quenching potential of different chemicals. The DPPH radical scavenging potential assay depends predominantly on the hydrogen atom-donating ability of a compound which confers on the compound that ability to stabilize DPPH radical, thus preventing its oxidative ability towards biomolecules [34]. Thus, the significant antioxidant activities of the fractions as well as the CE against DPPH radical infer that all the extracts have hydrogen atom-donating ability. This ability could be as a result of the presence of phenolic compounds in them. Though similar finding has been reported by Usunobun and Igwe, where the leaf extract also showed some level of scavenging activity [28]; however, in this study a very high level of radical DPPH scavenging activity (80.99 %) was exhibited. The difference in the studies could be due to differences in the reagent preparation, sample collection location, reagents quality and efficiency of instruments used. Contrary to DPPH assay, ferric reducing antioxidant potential assay depends largely on the electron-donating ability of a compound by reducing Fe^{3+} to Fe^{2+} in order to achieve stability of Fe. As a result, significant ferric reducing antioxidant potential showed by all the extracts could be attributed to their ability to donate electron which reduce Fe^{3+} to Fe^{2+}. Also, the antioxidant activity showed by the extracts in form of Fe reduction could be as a result of the presence of phenolic compounds which are known as anti-oxidative secondary metabolites [30].

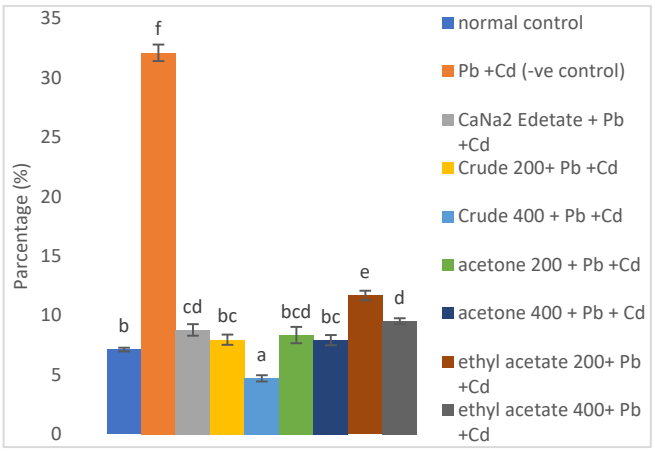

Fig. 16 Effect of CE and Fractions of *Pterocarpus mildbraedii* Leaf on Testes DNA Fragmentation in Cadmium and Lead Chloride Induced Toxicity in Male Albino Rats

Lipid peroxidation; a process in which free radicals extract electrons from cell membrane lipids (this mostly affects polyunsaturated fatty acids due to the presence of double bonds) has been described as an oxidative breakdown of lipids. This process of has been anticipated to ensue through a free radical chain reaction, which has been implicated in the damage/injury of cell biomembranes [35]. The damage caused has been established to predispose victims to numerous diseases viz; cardiovascular diseases, cancer and diabetes [36]. Inhibition of lipid peroxidation prevents the oxidative breakdown of lipids (where free radicals extract electrons from cell membrane lipids). The significant inhibitory effects exhibited by the extracts imply that the extract was able to sabotage free radical chain reaction and thus prevented oxidative breakdown of lipids in the egg yolk. The ability of the extracts to prevent lipids oxidative breakdown, similar to their DPPH scavenging potentials and ferric reducing antioxidant potential, could be accredited to the presence of phytochemicals in them. Although, AF recorded the highest amount of the compounds than the CE, the highest antioxidant activities showed by CE could be as a result of presence of different types of the phenolic compounds, most especially flavonoids in them as their activities have been shown to be structure-dependent [37].

Lead acetate and cadmium chloride enhanced the

intracellular formation of reactive oxygen species (ROS) causing damage of several organs. Under the influence of lead and cadmium, oxidative stress occurs via two different pathways: (1) The generation of ROS, such as hydrogen peroxide, singlet oxygen, and (2) the depletion of antioxidant reserves such as CAT, SOD and GSH [38], [39]. GSH has been reported to be the most abundant cellular sulphydryl molecule that interacts with oxidizing agents [40]. SOD neutralizes singlet oxygen and spontaneously dismutates superoxide radicals (O^{-}_{2}) to H_2O_2, while CAT decomposes H_2O_2 to O_2 and H_2O thus preventing lipid peroxidation [41]. Depletion of these antioxidant enzymes and molecules has been associated with oxidative stress. Oxidative stress is a physiological state when increased production of free radicals such as superoxide, hydroxyl radicals, singlet oxygen, hydrogen hydroxide and hydroperoxyl radicals, is compensated with decreased levels of antioxidant system [42]. These free radicals attack several biomolecules of all cells leading to loss of integrity of cellular and mitochondrial membranes as a result of these, membranes undergo increased process of lipid peroxidation [43]. Therefore, decreased levels of the antioxidant enzymes and molecules could be implicated in testicular damage. Oral administration of all the extracts at doses of 200 and 400 mg/kg body weight significantly increased the testicular activities of CAT and SOD as well as GSH level in the testis of the experimental male rats contrary to the untreated rats, although CE showed significantly higher levels than other extracts followed by AF and the least was EF. It may be inferred from the study that the extracts exhibited *in vivo* antioxidant activities against lead and cadmium-induced oxidative stress by elevating the levels of antioxidant system thus protecting the integrity of the testis. MDA is a marker for cell membrane damage. It is produced when the polyunsaturated fatty acids of the cell membrane undergo peroxidation. MDA is also reported to be one of the biomarkers of oxidative stress in clinical cases. Thus, increased level of MDA implies damage to the cell membrane of different cells and failure of antioxidant mechanisms to prevent excessive production of free radicals. As such, significant decrease in the level of MDA of the groups treated with the extracts implies that the extracts prevented peroxidation of the testicular cell membrane. The testicular-protective effects demonstrated by the extracts could be attributed to the secondary metabolites especially phenolic compounds present in the extracts as these compounds have been shown to exhibit antioxidant activities [29]-[31]. However, it is noteworthy that CE and AF exerted more protective effects than methanol and aqueous fraction as shown in the level of MDA. To the best of our knowledge, no study has reported testicular-protective effects of the CE or its fractions, however, studies have only reported hepato-protective effects of leaves extract of *Pterocarpus mildbraedii* [12], [44].

Estimation of testicular enzymes is one of the best methods of evaluating the toxicity of lead and cadmium on male reproductive organs. Significant increase in the level of LDH, ACP, ALP in the serum of negative control male rats in this study indicate the detrimental effects of both lead and cadmium in male reproductive organ of the rats. Exposure of mammals to lead and cadmium toxicities could lead to deterioration of some enzymes due to the interference with various sulfhydryl groups of such enzymes as well as metal-induced oxidative stress [45]. ALP is involved in the synthesis of nuclear proteins, nucleic acids and phospholipids, as well as the cleavage of phosphate esters. It also plays a role in mobilizing carbohydrates and lipid metabolites to be utilized either within the cells of the accessory sex structure or by the spermatozoa in the seminal fluid [43]. The elevated testicular ALP activity by the lead/cadmium chloride might be as a result of leakage of the enzymes in the testis. ACP is widely distributed in the testes and is important in the physiology of sperm [46]. The activity of LDH in the testicular tissues is associated with maturation of germinal epithelial layer of the seminiferous tubules. LDH is also found in Sertoli and spermatogenic cells and responsible for the testis energy production and biotransformation. Hence, inhibition of its activity in the Pb/Cd treated rats might be as a result of denaturation of spermatogenic cells as a result of testicular tissue denaturation. Nevertheless, significant reduction of these enzymes in the serum might signify reversion of the enzymes in the testicular tissue and other tissues which could be as a result of the ameliorative effects of the crude and fractions of *Pterocarpus mildbraedii* leaf extracts on the testicular tissues and other tissues where the enzymes are being hosted.

Prolonged exposure to cadmium and lead results in declining fertility in man and animals by reducing sexual hormones synthesis. Previous studies have shown that toxic metals such as lead and cadmium can modify hormone levels by affecting the hypothalamic–pituitary–testicular axis in different aspects. Pb and Cd affected the circadian pattern release of noradrenaline, a regulator of hypothalamus hormone secretion, which resulted in changes in the daily pattern of serum testosterone and LH levels [47]. The anterior pituitary secretes both FSH and LH; the latter via regulating testosterone secretion plays an important role in the final maturation of spermatocytes, while the former is needed for the maintenance of gametogenesis [48]. Assay of gonadotrophic hormones amongst untreated male rats exposed to lead acetate in the present study showed significant depression in the serum levels of FSH and LH. The reduced serum testosterone level also observed could be attributed to the reduction in the level of LH. LH is responsible for maintaining testosterone concentration [49]. Perhaps the reduction of gonadotropin secretion by lead acetate in the present study may be an account of a possible depressive effect on the hypothalamic neural mechanisms essential for the release of Gonadotropin Releasing Hormone [50]. This eventually will lead to disturbances in the secretion of pituitary gonadotropins which is essential for both spermatogenesis and steroidogenesis [51].

Treatment of lead acetate toxicity male rats with the crude and fractions of *Pterocarpus mildbraedii* leaf extracts ameliorated the detrimental effect of lead acetate on parameters under investigation as seen in the CE (400 mg/kg bw) and AF treated rats. There was reversion of the hormones levels towards fairly normal values comparable to values seen amongst untreated rats. The reversion of the hormones levels as

observed in the extract treated rats might be due to the presence of tannins, phenols and flavonoids in the extract which serve as antioxidant and prevent or terminate the lead/cadmium-induced oxidative stress [45]. These results are in agreement with previous reports that supplementation with ascorbic acid (antioxidant agent) reverted to normal levels of FSH, LH and testosterone following cadmium induced toxicity in male rats [52].

Although Pb/Cd-induced ROS production and resulting mitochondrial dysfunctions can mediate a series of testicular cell injuries, there must have been other mechanisms to provoke these effects. One of other proposed mechanism may be Pb/Cd direct inhibition of DNA repair [53]. ROS do not only damage the deoxyribose sugar by removing hydrogen atom but also change the DNA structure in the form of degradation of bases (purine and pyrimidine), break single and double bond. 8-hydroxydeoxyganiosine (8-0H-dG) is produced in this process. Haber Weiss and Fenton reaction also produce ROS and damage protein, carbohydrates and DNA [54]. Similarly, the reversal of increased fragmented DNA in the extract-treated groups may indicate stalled apoptosis, possibly due to the antioxidant properties of the phenolic groups in the extract [55].

V. Conclusion

It can be concluded from this study that the CE and fractions of *Pterocarpus mildbraedii* leaves possess testicular-protective properties against cadmium and lead toxicity. The CE exhibited higher testicular-protective activity than the fractions which might be as result of its vital phytochemical constituents as well as antioxidant activities. Therefore, *Pterocarpus mildbraedii* leaf extract can be explored further for the management of male infertility and lead/cadmium toxicity.

Acknowledgment

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