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D-3-O-methylchiroinositol (from *Pilostigma thonningii*) ameliorates cadmium chloride ($CdCl_2$)-induced toxicity in male reproduction

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ABSTRACT: This study examined the possible protective effect of D-3-O-methylchiroinositol isolated from stem bark of *Pilostigma thonningii* on cadmium chloride ($CdCl_2$)-induced reproductive toxicity in male Wistar rats. The results show that the immotile, headless and sluggish sperm count decreased from Month 2 to Month 3 respectively in the $CdCl_2$-challenged but D-3-O-methylchiroinositol-treated groups, and increased in the $CdCl_2$-only groups from Month 1 to Month 3. Cadmium (Cd) accumulated in the testes of Wistar rats and decreased sperm morphology including sperm count, sperm motility. It also increased immotile sperm count, headless sperm count and sluggish sperm count. However, in this study, D-3-O-methylchiroinositol which has a structural formula similar to the phosphatidylinositol phosphate with proven antioxidant potentials reversed these toxic effects by months 2 and 3. Histopathology results revealed that $CdCl_2$ significantly reduced the volume of spermatozoa in the seminiferous tubules and resulted in reduced spermatogenesis however, amelioration with D-3-O-methylchiroinositol restored the testicles to normal spermatogenic activities.

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Cadmium is a biologically accumulative non-essential element occurring in the environment (McLellan *et al.*, 1978; Nwokocha *et al*., 2012a, b). It is used traditionally in pigments, coatings and stabilizer production (NRC, 2007) and in industries for rubber processing, protective plating on steel, galvanizing, electrode material in nickel cadmium batteries, pigments in plastic and glass, production of pesticides, and as stabilizer for poly vinyl chloride (PVC) products (Kidambi *et al.,* 2003). The United Nations Environment Program listed cadmium along with other heavy metals, in her International Register of Potentially Toxic Chemicals (IRPTC, 1986). Cadmium has been ranked among the ten (10) most toxic compounds for human health (Hirano and Suzuki, 1996). Cadmium is also found naturally in tobacco leaves hence cigarettes smoking is a sure way to cadmium exposure of upto 1.7 µg cigarette^{-1} (Morrow, 2001; NTP, 2005). Cadmium is a potent heavy metal carcinogen to animals (Karimi *et al.*, 2014) and humans (Odewumi *et al.*, 2015). The reproductive potential of species and their survival have been threatened by an increased industrial and environmental contamination (Bu *et al.*, 2011). The gonads, ventral prostate, liver and kidney are target sites for cadmium toxicity in rodents (Haidry and Malik, 2014; Abdel Moneim *et al.*, 2014; Nair *et al.*, 2015; Baiomy, 2016). It has been reported to exert its genotoxicity via the production of reactive oxygen species (ROS) and by inhibiting cell proliferation and DNA replication (Nair *et al.*, 2015).

Although the human body has natural antioxidant system to detoxify free radicals, exposure of the body to several exogenous chemicals can cause generation of free radicals to exceed the protective capacity of the antioxidants, thereby resulting in oxidative stress (Wu and Cederbaum, 2003). Several industrial chemicals, including cadmium chloride ($CdCl_2$), have been reported to cause oxidative stress in various cells and organs of the body (Valko *et al.*, 2005). Its toxicity has been reported in various body organs including; the kidney, lungs, stomach, brain, bone, blood, liver, heart, testes and ovaries (Valko *et al.*, 2005). Cadmium has been reported to be carcinogenic (Koyama *et al.*, 2002), and cause histopathological damages on the male reproductive organs (Massanyi *et al.*, 2007). D-3-O-methylchiroinositol isolated from the stem bark of

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Piliostigma thonningii has a structural formula similar to the phosphatidylinositol phosphate, which participates in the insulin signaling pathways that stimulate glucose transport (Asuzu and Nwaehujor, 2013) and is known to possess antioxidant activities (Asuzu and Nwaehujor, 2013). It has been observed that D-chiroinositol reduced urinary potency with impaired glucose tolerance, insulin resistance and type 2 diabetes mellitus in rhesus monkeys and human subjects (Kennington *et al*., 1990). D-chiroinositol is also known to improved glucose tolerance in normal rats and increased gluconeogenesis in the diaphragm (Ortmeyer *et al*., 1993). The increased effort in the development of an effective anti-cancer drug as a result of the challenge posed by irreversible inhibition of spermatogenesis by toxic chemicals due to industrial exposure have encouraged this research. The objective of this article is to investigate the possible protective effect of D-3-O-methylchiroinositol isolated from stem bark of *Pilostigma thonningii* on cadmium chloride ($CdCl_2$)-induced reproductive toxicity in male Wistar rats.

MATERIALS AND METHODS

D-3-O-methylchiroinositol was isolated from the stem bark of *Piliostigma thonningii* as described by Asuzu *et al.* (1999). The stem bark of the plant was exhaustively extracted with 80% methanol in a soxhlet at 40 °C for 12 h. The pure compound was isolated using column and TLC, lyophilized and stored in the fridge at 4°C until used for the experiments (Asuzu and Nwaehujor, 2013).

*Chemicals***:** Cadmium chloride ($CdCl_2$), Tween20 used for this study were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). All other reagents were of analytical grade.

*Laboratory animals***:** Thirty-six (36) adult male albino Wistar rats (10 weeks old) and weighing 170 -190 g were obtained from the animal house of the Department of Biochemistry, College of Medical Sciences, University of Calabar, Nigeria. They were acclimatized for 7 days and were allowed *ad libitum* access to feed and water. Experimental animals were kept in accordance with the guidelines for animal care as contained in the animal ethics handbook of the Faculty of Basic Medical Sciences, University of Calabar, Nigeria.

*Experimental design***:** The rats were randomly assigned to 1 of 3 groups (n = 12) as follows; A: control, B: $CdCl_2$ only group, C: $CdCl_2$+D-3-O-methylchiroinositol group. Group A rats were orally administered distilled water only, group B rats received $CdCl_2$ (2.5 mg/kg b.w. in drinking water), group C rats were treated with D-3-O-methylchiroinositol (2 mg/kg b.w./daily) and $CdCl_2$ (2.5 mg/kg b.w. day) in drinking water. Cadmium chloride ($CdCl_2$) was dissolved in the drinking water at a dose of 2.5 mg/kg and D-3-O-methylchiroinositol were dissolved in 0.5 % Tween20 and administered per os for 90 days. The dose for $CdCl_2$ was chosen from previous studies by El-Demerdash *et al.* (2004) and Alkhedaide *et al.* (2016) for cadmium chloride, and Asuzu and Nwaehujor (2013) for D-3-O-methylchiroinositol. The chosen dose for cadmium chloride was shown to cause significant oxidative stress in various tissues of the body (El-Demerdash *et al.*, 2004; Alkhedaide *et al.*, 2016) while that of D-3-O-methylchiroinositol showed significant antioxidant effect (Asuzu and Nwaehujor, 2013). After every 4 weeks, 4 animals from each group was humanely euthanized under chloroform anesthesia. The experiment lasted for 3 months.

Semen collection: The testes were immediately exteriorized through a mid-caudoventral abdominal incision with sterile scalpel blade. Sperm cells were then collected from the caudal epididymis. (Oyeyemi *et al.,* 2011).

Sperm count: This was done by removing the caudal epidydimis from the right testes and blotting with filter paper. The caudal epidydimis was immersed in 5ml formal-saline in a graduated test tube and the volume of fluid displaced was taken as the volume of the epidydimis. The volume of the epidydimis and the caudal epidydimis were poured into a ceramic mortar and homogenized into a suspension from which the sperm count was carried out using the improved neubauer haemocytometer under the microscope. (Zemjanis, 1977).

*Sperm motility***:** A small drop of sperm suspension was collected with fluid from the caudal epidydimis via scapel and dropped onto a slide. The diluents (buffered 2.9% sodium citrate solution) kept at 37 degrees Celsius was added to the sperm suspension until the desired dilution was obtained. Sperm motility was assessed by the method described by Zemjanis (1977). The motility of the epidydimal sperm was evaluated microscopically 2-4 minutes of their isolation from caudal epidydimis and later expressed as percentages.

Sperm liveability: This was assessed by adding 2 drops of warm eosin nigrosin stain to the semen on a pre warmed slide, a uniform smear was made and dried with air and the stained slide was immediately examined under the microscope using x400 magnification. The live sperm cells were unstained while the dead sperm absorbed the stain. The stained

and unstained were counted and the percentage calculated. (Oyeyemi *et al.,* 2011).

Testicular Histopathology: Testes of each rat were fixed in bouins fluid, passed through ascending series of ethanol and then through xylene and embedded to paraffin wax. The tissues were sectioned at the thickness of haematoxylin and eosin and mounted. All sections were examined under light microscope in x100 and x400 magnification. Photomicrographs of the lesions were taken for observation and documentation of histopathologic lesions.

Data analysis: The mean and standard error of mean were calculated for the semen characteristics and hormonal assay and were presented in percentages. One-way ANOVA (one-way analysis of variance) and Duncan multiple comparison test of the statistical package for social science (SPSS) were used to establish any significant difference at 95% confidence interval. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Spermatogenesis is a complex series of differentiation process that can be interfered by toxic chemicals, heavy metals, heat, radiation, deficiencies of hormones and immunodeficiency (Akunna *et al.*, 2012; Akunna *et al.*, 2014; Khanna *et al.*, 2016). In this study, results show a significant ($p>0.01$) reduction in spermatozoa count, spermatozoa motility, number of morphologically normal spermatozoa and a significant ($p>0.01$) increase in the number of morphologically abnormal spermatozoa in animal models exposed to cadmium, when compared to control and D-3-O-methylchiroinositol-treated groups (Fig. 1-5). The results in Figure 1 shows that the sperm count for group B and group C was significantly lower ($p< 0.05$) than group A value with group B having the lowest values across the months.

The mean motile sperm count and liveability followed the same pattern as mean sperm count showed values which were significantly lower ($p<0.05$) than the control group A value with group B having the lowest values across the months (Figure 2).

The results in Figure 3 shows that the immotile sperm count for group B was significantly higher ($p< 0.05$) than group A value and group B with group B having the lowest values across the months. The results in Figure 4 showed that the sluggish sperm count for group B and group C was significantly lower ($p< 0.05$) than group A value in the first month, but group B increased in values in months 2 and 3 significantly.

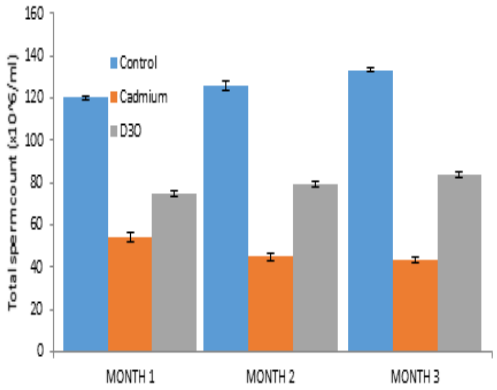

Fig 1: Effect of D-3-O-methylchiroinositol in cadmium chloride-induced toxicity on total sperm count.

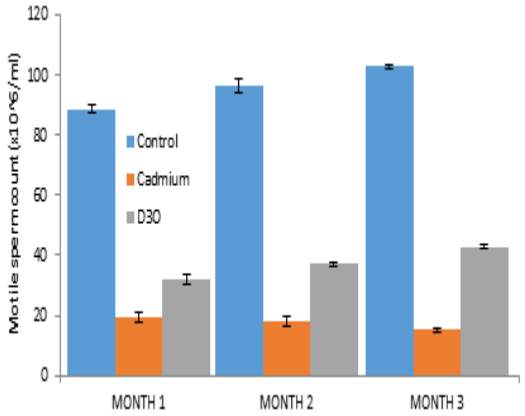

Fig 2: Effect of D-3-O-methylchiroinositol in cadmium chloride-induced toxicity on motile spermcount.

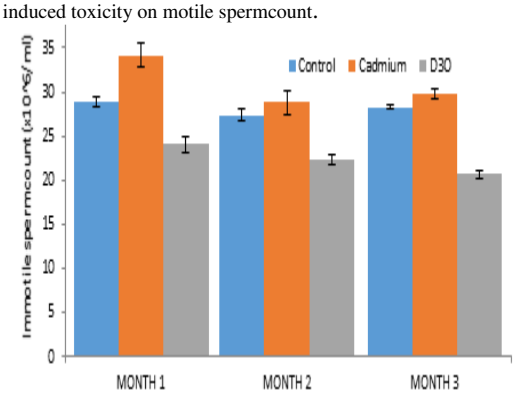

Fig 3: Effect of D-3-O-methylchiroinositol in cadmium chloride-induced toxicity on immotile sperm count.

The results in Figure 5 showed that the headless sperm count for group B and group C was significantly lower ($p< 0.05$) than group A value in the first month with group C having the highest headless sperm count. In months 2 and 3, group B had the highest count especially in month 3 which was significant

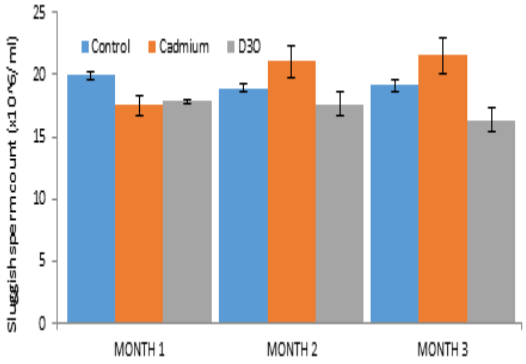

Fig 4: Effect of D-3-O-methylchiroinositol in cadmium chloride-induced toxicity on sluggish sperm count.

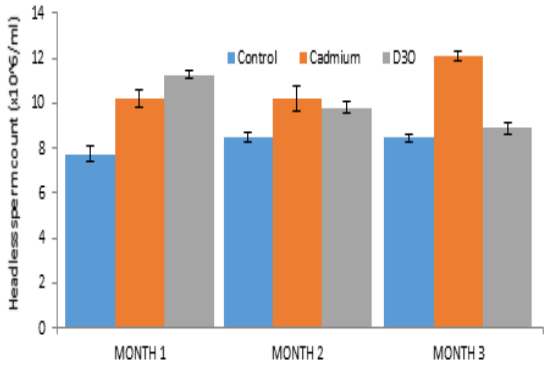

Fig 5: Effect of D-3-O-methylchiroinositol in cadmium chloride-induced toxicity on headless sperm count.

Our results are in line with several other studies which showed a degenerative ability of cadmium to testicular and epididymal tissues thereby contributing to male infertility via reducing sperm quality in humans and rats (Akunna *et al.*, 2014; Khanna *et al.*, 2016; Mendiola *et al.*, 2011; Benoff *et al.*, 2000; Toman *et al.*, 2008; De Souza Predes *et al.*, 2010; Benoff *et al.*, 2009; Xu *et al.*, 2001; Roychoudhury *et al.,* 2010). Cadmium has been reported to cause spermatotoxicity either by resulting in disruption of hypothalamic-pituitary axis or by direct effect on spermatogenesis through oxidative damage (Xu *et al.*, 2001; Roychoudhury *et al.*; 2010). Since sperm cell and testicular leydig cell mitochondria are common body cells that are susceptible to cadmium-induced oxidative stress the latter represents the major factor (Khanna *et al.*, 2016).

Cadmium cytotoxicity depends majorly on ionic mimicry distinct by calcium and zinc substitution which results in protein breakdown and ultimately endoplasmic reticulum stress and cell death (Sachdev and Davies, 2008). Several studies indicating the exact progression of mitochondrial dilapidation as a result of its cadmium toxicity has been reported (Sachdev and Davies, 2008). These includes opening of the mitochondrial permeability transition pore (mPTP) and Mitochondrial Calcium Uniporter (MCU) thereby leading to the release of cytochrome C into the cytosol. Unlike somatic cells, mammalian sperm cells present unusually high specific lipidic composition with high content of poly-unsaturated fatty acids, plasmalogenes and sphingomyelins responsible for its flexibility and the functionality. However, these lipids are substrates for peroxidation that may provoke severe functional disorder of sperm (Sachdev and Davies, 2008). Other studies have also shown that cadmium accumulates in target tissues (Nwokocha *et al.*, 2011, 2012a, b), resulting in significant oxidative stress in these tissues (Farombi *et al.*, 2012). Since cadmium absorption and excretion is reported to be slow, it can accumulate over time and cause toxic effects (McLellan *et al.*, 1978). The testes is one of the target tissues affected by cadmium (Samuel *et al.*, 2011). Therefore, it will be worthwhile to assess the likelihood of cadmium accumulation in the testes after a long-term exposure.

From histological diagrams, testicular interstitial cell showed a high degree of sloughing off and interstitial edema in the $CdCl_2$ treated group (Figure 6) throughout the period of the study. The seminiferous tubules did not show any visible lesions in the germinal epithelium but there was significant reduction in the accumulation of spermatozoa in the lumen of the seminiferous tubules (Figure 7). There was improved interstitial cell integrity in the D-3-O treated and the control group the germinal epithelial cells did not show any signs of degeneration (Figure 8). The study showed direct and indirect effects of $CdCl_2$. It is direct since Cd^{2+} replaces Ca^{2+} and Zn^{2+} by mimicking their physiological processes in the cells (Valko *et al.*, 2005), and indirect since Cd in non-reproductive glands (e.g. the hypothalamus and pituitary) negatively affects reproductive function through suppressed release of FSH and LH (Hoyer, 2005). Clinical (Varga *et al.*, 1993) and experimental (Massanyi *et al.*, 2007) data have reported that Cd accumulates in the testes following oral exposure. Treatment with D-3-O-methylchiroinositol offered better protection against Cd accumulation in this study, compared to the untreated groups. According to the trend our results, it is likely that three mechanisms are involved in D-3-O-methylchiroinositol's suppression of Cd accumulation in the testes. It is likely that D-3-O-methylchiroinositol (a) altered $CdCl_2$ absorption in the gut or (b) enhanced its excretion through the kidneys or (c) act as an antioxidant in the xenobiotic degradation or excretion of Cd.

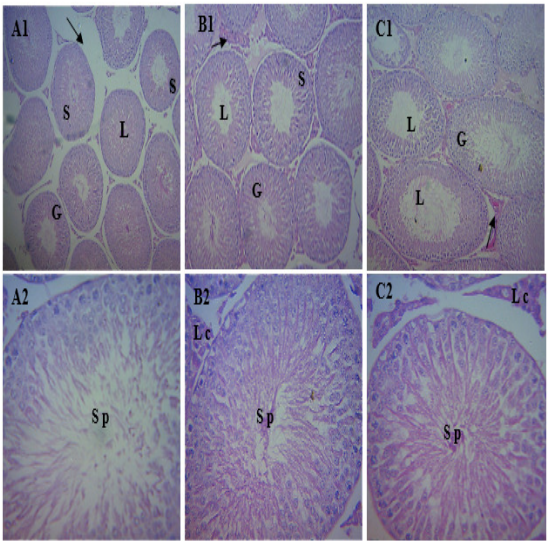

Fig. 6: The histology of Wistar rat testis, showing: (**A1** and **A2**) treated with $CdCl_2$; (**B1** and **B2**) $CdCl_2$ and D-3-O-methylchiroinositol and (**C1** and **C2**) controlled group treated with distilled water. The interstitial cells in (**A1**) indicated by the arrow are sloughed off, with fewer Leydig cells (**Lc**). The seminiferous tubules (**S**) integrity is compromised with no visible lesion of the germinal epithelium (**G**), with accumulation spermatozoa (**Sp**) in the seminiferous tubules lumen (**L**). Note the improved interstitial cells integrity in (**B1**) compared with (**A1**) and the control (**C1**). The spermatozoa in (**A2**) are apparently without visible lesions compared with (**B2**) and (**C2**). H&E; X100 and X400.

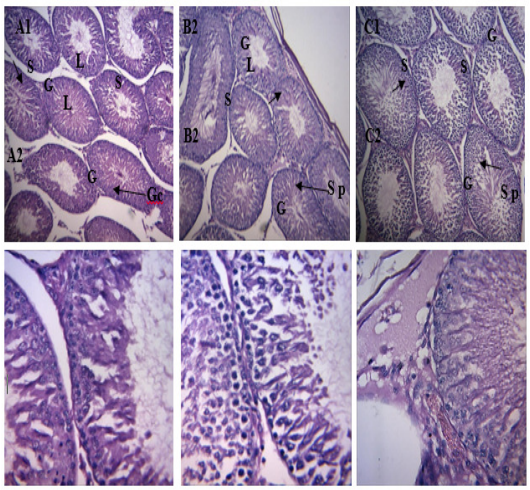

Fig 7: The histology of Wistar rat testis, showing: (**A1** and **A2**) treated with $CdCl_2$; (**B1** and **B2**) $CdCl_2$ and D-3-O-methylchiroinositol and (C1 and C2) controlled group treated with distilled water. Note the seminiferous tubules (**S**) in (**A1** and **A2**). For month two, the interstitial cells in (**A1**) indicated by the arrow are sloughed off, with fewer Leydig cells (**Lc**) as seen in month one. The seminiferous tubules (**S**) integrity is compromised with no visible lesion of the germinal epithelium (**G**), although the accumulation of spermatozoa (**Sp**) in the seminiferous tubules lumen (**L**) for (A1) is fewer than seen in (**B1** and **C1**). Note the improved interstitial cells integrity in (**B1**) compared with (**A1**) and the control (**C1**). The germinal epithelial cells (**Gc**) in (**A2**) are apparently degenerating compared to (**B1** and **C2**). H&E; X100 and X400.

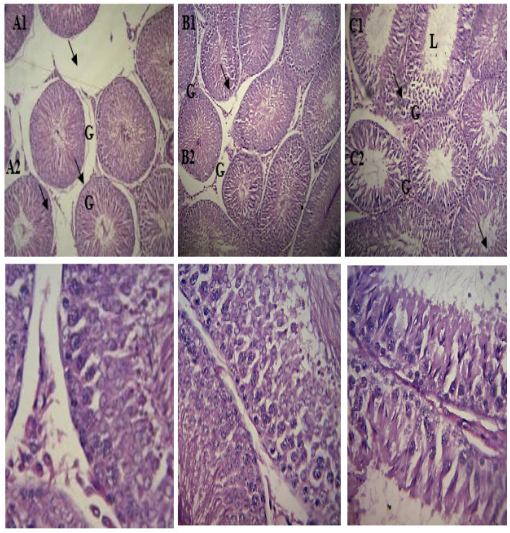

Fig 8: The histology of Wistar rat testis, showing: (**A1** and **A2**) treated with $CdCl_2$; (**B1** and **B2**) $CdCl_2$ and D-3-O-methylchiroinositol and (C1 and C2) controlled group treated with distilled water. For month three, the interstitial cells in (**A1**) indicated by the arrow are sloughed off, with fewer Leydig cells (**Lc**) as seen in month one and two with apparently no visible severity under light microscopy. The seminiferous tubules (**S**) integrity is compromised with no visible lesion of the germinal epithelium (**G**), although the accumulation of spermatozoa (**Sp**) in the seminiferous tubules lumen (**L**) for (A1) is fewer than seen in (**B1** and **C1**). Note the improved interstitial cells integrity in (**B1**) compared with (**A1**) and the control (**C1**). H&E; X100 and X400.

Conclusion: This study has provided an addition to the body of knowledge by extensively reporting the possible ameliorative mechanism pathway of D-3-O-methylchiroinositol in cadmium-induced spermatoxicity through assay of sperm count and morphology. The histopathology results revealed that $CdCl_2$ significantly reduced the volume of spermatozoa in the seminiferous tubules and resulted in reduced spermatogenesis. However, amelioration with D-3-O-methylchiroinositol restored the testicles to normal spermatogenic activities in male Wistar rats challenged with $CdCl_2$ toxicity.

Disclosure statement: The data reported in this study have not been submitted or published elsewhere.

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*Conflict of interest***:** The authors declare that there are no conflict(s) of interest.

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