ORIGINAL ARTICLE

Osteocyte viability and bone density in cadmium chloride-induced osteoporosis ameliorated with *Pilostigma thonningii* stem bark-extracted D-3-O-methy-chiroinositol

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Abstract

Background: This study examined the ameliorative effect of D-3-O-methyl-chiroinositol, isolated from the stem bark of *Piliostigma thonningii*, on cadmium chloride-induced osteoporosis in male Wistar rats.

Methods: Thirty-six rats were assigned to three treatment groups (n = 12). Group A (2 mL distilled water), group B: (2.5 mg/kg b.w. $CdCl_2$) and group C: (2.5 mg/kg b.w. $CdCl_2$ and D-3-O-methyl-chiroinositol 2 mg/kg b.w.). Bone ash, calcium, phosphate, magnesium, and zinc content, as well as bone histological changes were determined at the end of months 1, 2, and 3.

Results: There were significant differences ($P \leq 0.05$) in the weight of the cervical, tibia, and femoral bones in all groups. The serum concentration of $CdCl_2$ was significantly different across the three groups with time. There was significant variation ($P < 0.005$) in the mean bone ash across groups. The concentration of OH-proline was significantly different ($P < 0.0001$) across groups. There were significant differences ($P < 0.0001$) in bone calcium, magnesium, zinc, and phosphorus concentrations. Histology revealed high levels of bone mineralisation in the $CdCl_2$-treated group, indicative of osteoporosis with hypertrophied osteocytes, while the femur of Wistar rats treated with D-3-O-methyl-chiroinositol showed bone trabeculae and viable osteocytes.

Conclusion: The study concluded that D-3-O-methyl-chiroinositol extract from *Piliostigma thionningii* stem bark ameliorated cadmium chloride-induced osteoporosis in male Wistar rats.

KEYWORDS
cadmium chloride, D-3-O-methyl-chiroinositol, osteocytes, osteoporosis, toxicity, amelioration

1 | INTRODUCTION

Osteoporosis, defined as low bone mineral density (BMD), is a public health concern as it may increase the risk of fractures, morbidity/mortality, and health care costs.[1] It is characterised by the deterioration of the architectural design of bone tissues which reduces bone tissue mass, making the bone become fragile and fracture easily.[2]

In addition to the genetic, nutrition, lifestyle, and mechanical factors that determine bone density, environmental exposure to toxic heavy metals such as cadmium (Cd), chromium, and lead also plays a role in osteoporosis.[1] Cadmium toxicity has been reported in several organs including the bone.[3] Cadmium, which has a biologically accumulative effect in the body, occurs traditionally in the environment as a non-essential element used in pigments, coating and stabilizers.[4-7] It is also used in production of pesticides, galvanising and steel protective plating, rubber processing, batteries, and polyvinyl chloride (PVC) production,[8] and has been listed by the United Nations Environmental Program as a potentially toxic chemical.[9] Cigarette smoking commonly exposes individuals to cadmium as it is naturally found in tobacco leaves,[10] and cadmium has been reported as a major heavy metal carcinogen in humans and animals.[11]

Most industrial chemicals, including cadmium chloride ($CdCl_2$), cause oxidative stress in the body.[3] Cadmium toxicity is believed to result from the production of reactive oxygen species (ROS) and also from inhibition of cell proliferation and replication of DNA,[12] which may eventually lead to oxidative stress. For many years, researchers have believed that cadmium exposure was most damaging to various visceral organs,[3,7,12-14] and bone loss was secondary. A serious bone disease found in the Jinzu River basin of Japan first hinted that cadmium might cause serious bone loss. Itai-itai disease, which means "ouch, ouch," is a painful result of chronic cadmium poisoning from mining byproducts dumped upstream.[15] These patients had extreme bone demineralization. Clinically significant bone lesions usually occur late in severe chronic cadmium poisoning and include pseudofractures and other effects of osteomalacia and osteoporosis.[16] Cadmium is known to release calcium from bone within hours of exposure[17] and its cytotoxicity depends majorly on distinct ionic mimicry by calcium and zinc substitution, resulting in protein breakdown and ultimately endoplasmic reticulum stress and mitochondrial dilapidation, and eventually cell death.[18]

D-3-O-Methyl-chiroinositol, isolated from the stem bark of *Piliostigma thonningii* has a structural formula similar to phosphatidylinositol phosphate, which participates in the insulin signaling pathways that stimulate glucose transport, and is known to possess strong antioxidant activities.[19] It has been observed that D-3-O-methyl-chiroinositol reduces urinary potency with impaired glucose tolerance, insulin resistance and type 2 diabetes mellitus in rhesus monkeys and human subjects.[20] The challenges in developing novel therapeutic compounds to ameliorate the toxic effects of cadmium exposure on bone led to this research.

2 | METHODS

2.1 | Extraction and purification of D-3-O-methyl-chiroinositol

D-3-O-methylchiroinositol (D3O) was isolated from the stem bark of *Piliostigma thonningii*, as described by Asuzu et al.[21] The stem bark of the plant was exhaustively extracted with 80% methanol in a Soxhlet extractor at 40°C for 12 hours. The pure compound was isolated using a column and TLC, lyophilized and stored in the fridge at 4°C until used for the experiments.[19]

2.2 | Chemicals

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

2.3 | Laboratory animals

The conduct of the research was approved and in accordance with the approved research guidelines on laboratory animal use of the Faculty of Basic Medical Sciences, University of Calabar, where the animal study was carried out. All animals were humanely handled and their welfare respected throughout this study as stipulated in the 1964 Helsinki Declaration, as amended.[22] Thirty-six 10-week-old male Wistar rats weighing 170-190 g were obtained from the laboratory unit of the Department of Biochemistry, College of Medical Sciences, University of Calabar. The rats were maintained at an ambient temperature between 28 and 30°C, with a humidity level of 55 ± 5%, and a standard (natural) photoperiod of approximately 12 hours of light (6:30 AM-18:30 PM) alternating with approximately 12 hours of darkness (18:30 PM-6:30 AM). Prior to the experiment, the rats were acclimatised for 7 days with *ad libitum* feed and water. Afterwards the rats were randomly assigned to 3 groups (n = 12): group A (control) was orally administered 2 mL distilled water); group B was administered 2.5 mg/kg b.w. $CdCl_2$ in drinking water;[23] group C was administered 2.5 mg/kg b.w. $CdCl_2$ in drinking water and D-3-O-methyl-chiroinositol (D3O) at 2 mg/kg b.w. daily dissolved in 0.5% Tween20 and administered per os.[19] The experiment lasted for 3 months. Four animals from each group were humanely euthanized at the end of each month under chloroform anaesthesia; the femur, tibia and cervical bones were harvested. Volumetric bone density of the femur and tibia was determined by Archimedes' principle. After degassing the bone in deionized water, the left femur and tibiae were cut at 2.5 and 2.5 mm, respectively, from the midshaft to obtain a 5-mm-long diaphyseal bone section, which essentially consisted of cortical bone for measurement of bone calcium, phosphate, magnesium,

and zinc content. Bone tissues were also obtained from the left tibial bones, the left femoral bones and the cervical bones and were fixed in Bouin's fluid for histological study.

2.4 | Bone ash determination

At the end of months 1, 2, and 3, the right femurs and tibiae of selected animal were placed in a muffle furnace at 700°C for 20-24 hours. Crucibles were cooled in a 100°C oven for 60 minutes before weighing with a sensitive Shimadzu® digital electronic weighing balance (Model BR9010; Guangdong, China).

2.5 | Measurement of bone calcium, phosphate, magnesium, and zinc

The 5 mm left femoral and tibial bones tissues obtained were used for measurement of calcium, phosphate, magnesium, and zinc content. After washing with ethanol, each 5-mm piece of tibial diaphysis was dried at 60°C for 1 hour and weighed. Then, 5 mL of hydrochloric acid and 3 mL of nitric acid were added, dissociation by heating was performed with a sand burst, and the mixture was dissolved in 50 mL of purified water. Next, a 10-fold dilution was performed and 3 standard solutions were prepared (calcium: 0, 8, and 40 ppm; phosphate: 0, 8, and 40 ppm; magnesium: 0, 0.4, and 2 ppm; and zinc: 0, 0.02, and 0.1 ppm, respectively). The contents of each sample were determined using an atomic absorption spectrophotometer (Shimadzu, Japan) and calcium (Ca), phosphate (P), magnesium (Mg) and zinc (Zn) contents of dry bone weight were expressed as percentages.

2.6 | Chemical analyses

Following biomechanical assessment, pieces of bones from the left femur of all groups in the experiment were demarrowed with cold 0.25 mol/L sucrose, pulverized in liquid nitrogen, lyophilized, and diluted in 6 mol/L HCl (1:5 wt:vol), then analyzed colorimetrically for hydroxyproline using Ehrlich's reagent.
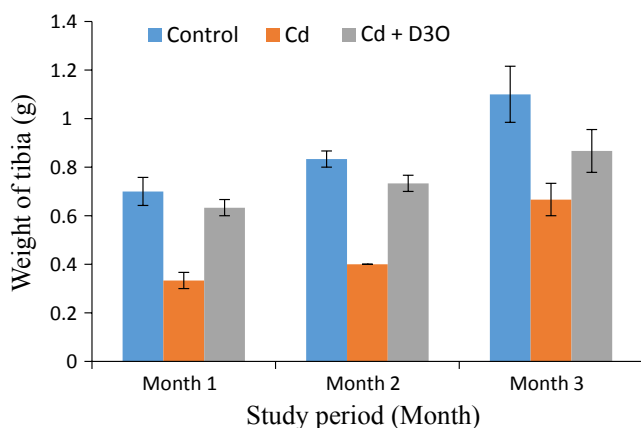
2.7 | Histological examination

The femur, tibia and cervical bones fixed in Bouin's fluid were decalcified, dehydrated in increasing concentrations of ethanol, cleared in xylene before being embedded in paraffin wax. Sections of 5 μm thickness were obtained with a rotary microtome (Model 1512; Leitz®, Wetzlar, Germany) and mounted on clean glass slides. The sections were then stained with hematoxylin and eosin and studied with a light microscope as described by Bancroft and Stevens.[24] Images were then captured and viewed with a Moticam Camera Motic Image Plus 2.0 (Motic China group Ltd, Xiemen, China).

2.8 | Biochemical analysis

Cadmium concentrations in the bones were determined using the femur, tibia, and cervical bones. Briefly, samples of the bones obtained at the end of months 1, 2, and 3 of the experiment were dried to a constant weight. Then they were digested in concentrated nitric acid. Once the digestion was complete, the samples were cooled at room temperature and brought to a constant volume (5 mL) by adding deionized water. For cadmium determination, an atomic absorption spectrophotometer (Perkin-Elmer Analyst 100) was used.
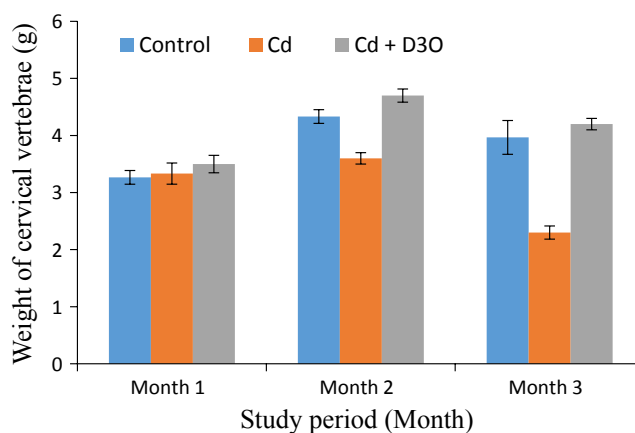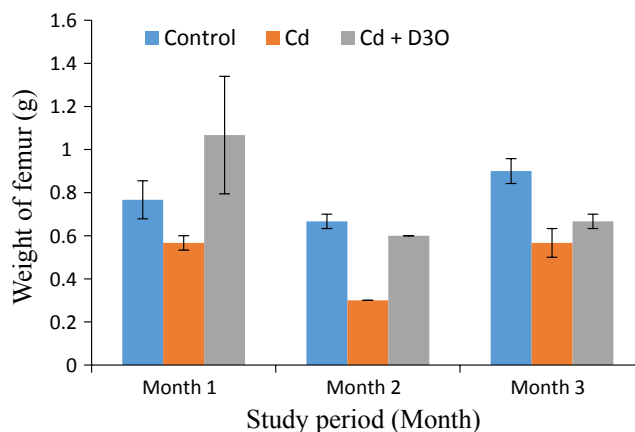
FIGURE 2 Mean changes in cervical bone weight

FIGURE 1 Mean changes in tibia bone weight

FIGURE 3 Mean changes in femoral bone weight

2.9 | Data analysis

The mixed design ANOVA model was used to determine significant differences in mean bone density and serum concentration of normal, negative and D-3-O-methyl-chiroinositol (D3O) ameliorated groups at months 1, 2, and 3, respectively. The Greenhouse-Geisser adjustment was reported where Mauchley's test of sphericity failed; assumption of sphericity, and main effects were compared with Bonferroni adjustment. Values of $P < 0.05$ were considered significant.

3 | RESULTS

There were statistically significant differences ($P \leq 0.05$) in the weights of the tibial, cervical and femoral bones in all groups (Figures 1-3). The result shows a statistically significant decrease in the weight of the bones measured in animals administered cadmium chloride only, while the group ameliorated with D-3-O-methyl-chiroinositol (D3O) showed significant recovery of bone weight when compared with the control administered with distilled water only.

TABLE 1 Variation in serum cadmium chloride concentration across groups

Group	Variable	Time (mo)	Mean	SE	Time (mo)	Mean difference	P
A. Control (normal)	CdCl$_2$	1	0.013	0.013	1	−1.393	<0.0001
		2	0.023	0.006		−1.07	<0.0001
		3	0.02	0.004		0.323	<0.0001
B. CdCl2 (treated)		1	1.407	0.013	2	−1.413	<0.0001
		2	1.437	0.006		−1.03	<0.0001
		3	1.447	0.004		0.383	<0.0001
C. D3O (treated)		1	1.083	0.013	3	−1.427	<0.0001
		2	1.053	0.006		−1.017	<0.0001
		3	1.037	0.004		0.41	<0.0001

TABLE 2 Variation in bone ash across groups

Group	Variable	Time (mo)	Mean	SE	Time (mo)	Mean difference	P
A. Control (normal)	Bone ash	1	0.373	0.006	1	0.162	<0.0001
		2	0.373	0.006		−0.013	0.544
		3	0.397	0.003		−0.175	<0.0001
B. CdCl2 (treated)		1	0.211	0.006	2	0.162	<0.0001
		2	0.211	0.006		−0.009	>0.05
		3	0.215	0.003		−0.17	<0.0001
C. D3O (treated)		1	0.386	0.006	3	0.182	<0.0001
		2	0.381	0.006		0.007	0.314
		3	0.39	0.003		−0.175	<0.0001

TABLE 3 Variation in hydroxyproline concentration across groups

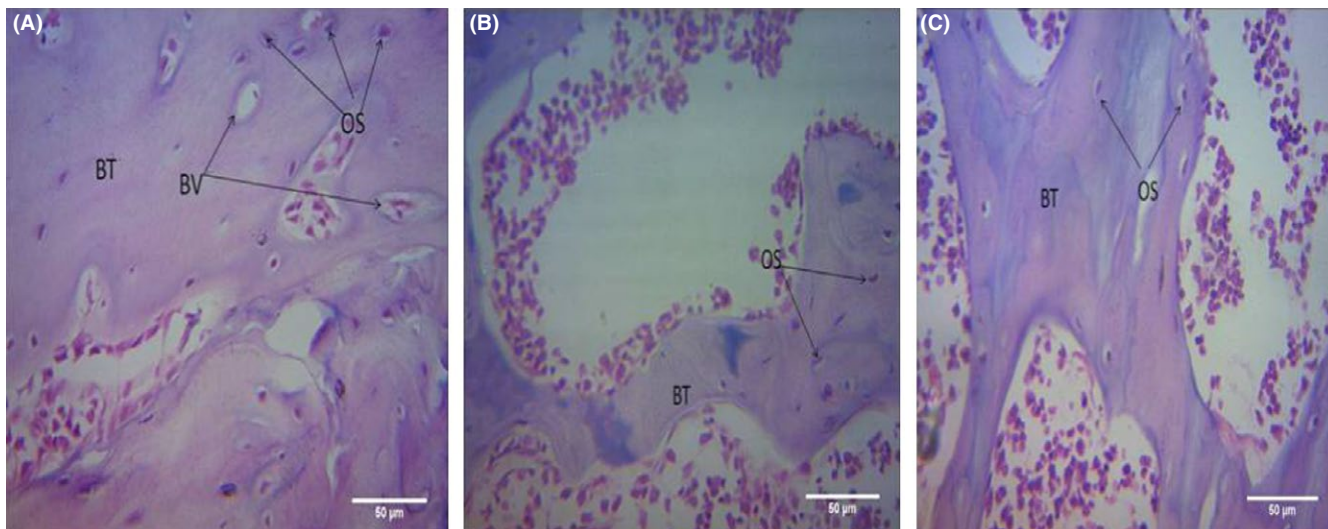| Group	Variable	Time (mo)	Mean	SE	Time (mo)	Mean difference	P
A. Control (normal)	OH-Pro	1	85.33	0.816	1	24.0	<0.0001
		2	85.33	1.0		18.667	<0.0001
		3	87.33	0.816		−5.333	0.011
B. CdCl2 (treated)		1	61.33	0.816	2	24.0	<0.0001
		2	61.33	1.0		13.0	<0.0001
		3	51.33	0.816		−11.0	0.001
C. D3O (treated)		1	66.67	0.816	3	36.0	<0.0001
		2	72.33	1.0		11.667	<0.0001
		3	75.67	0.816		−24.333	<0.0001

FIGURE 4 Photomicrograph of the femur of a Wistar rat at month 1. A, Group treated with $CdCl_2$, showing bone trabeculae (BT) and osteocytes (OS). B, Group treated with D-3-O-methyl-chirioinositol. Note the blood vessel (BV). C, Control group. Also observe the osteocyte (OS) and the trabeculae (BT). There was no visible lesion in the treated groups for the period of 1 mo compared to the control group
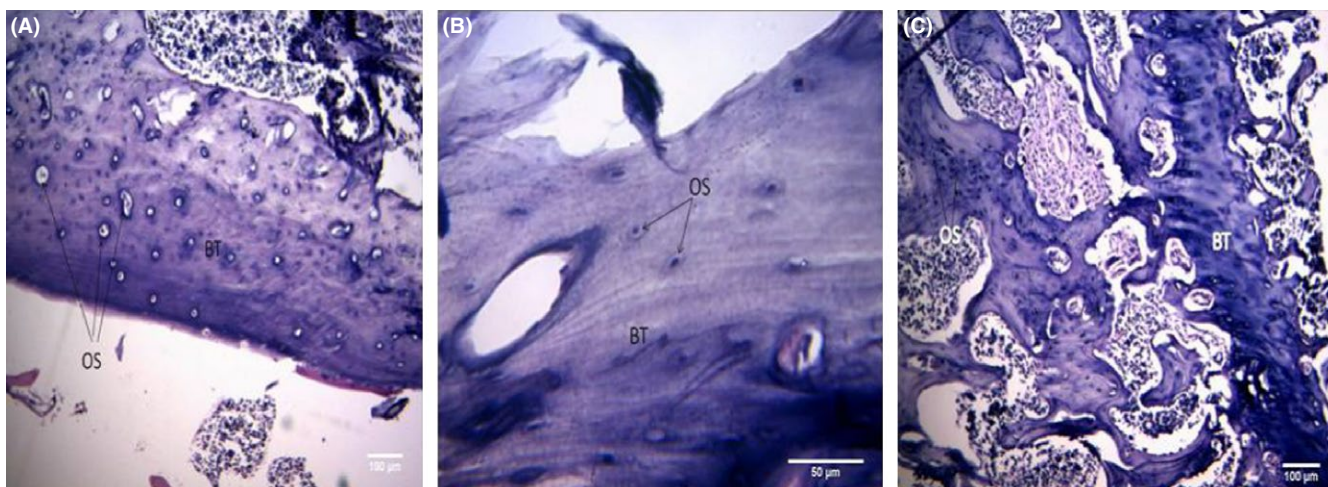

FIGURE 5 Photomicrograph of the femur of a Wistar rat at month 2. A, Group treated with $CdCl_2$. A considerable number of the lacunae in the bony mass are hypertrophied enclosing necrotic cells. B, Group treated with DBO, showing the bone trabeculae (BT) and osteocyte (OS) in apparently normal lacunae. C, Control group. There were no visible lesions seen in the control group rat

The repeated measures ANOVA test revealed that the tibial bone weight (Figure 1) varied significantly over the 3 months of study ($F_{1.129,6.775} = 26.98$; $P = 0.001$). However, the interaction between time and experimental groups was not found to be significant ($F_{2.258,6.775} = 0.776$, $P > 0.005$). The weight of the cervical bone (Figure 2) varied significantly ($P = 0.001$) across groups for the 3 months of the study ($F_{2,12} = 24.054$, $P < 0.0001$) and within the three groups with time ($F_{4,12} = 10.423$; Figure 2). Femoral bone weight also varied significantly ($P = 0.033$) over the period of study ($F_{1.175,7.051} = 6.653$; Figure 3). The serum concentration of cadmium chloride (Table 1) did not show any significant variation ($P > 0.005$) across the 3 months of the experiment ($F_{1.059,6.356} = 0.186$; $P = 0.695$),

but was significantly different across the three experimental groups with time ($F_{2.119,6.356} = 8.412$; $P = 0.016$). There were significant differences ($P < 0.005$) in the mean bone ash (Table 2) across the groups during the 3 months of the study ($F_{2,12} = 10.257$; $P = 0.003$) and between the three experimental groups with time ($F_{4,12} = 3.246$; $P = 0.051$). The serum concentration of hydroxyproline (OH-Pro; Table 3) was not significantly different within groups across the 3 months of experiment ($F_{2,12} = 3.529$; $P = 0.062$), but was significantly different across the three experimental groups with time ($F_{4,12} = 30.271$; $P < 0.0001$). There was no significant difference in the experimental group measures in calcium concentration over the 3 months ($F_{1.02,6.121} = 0.605$; $P = 0.469$), nor in the interaction between
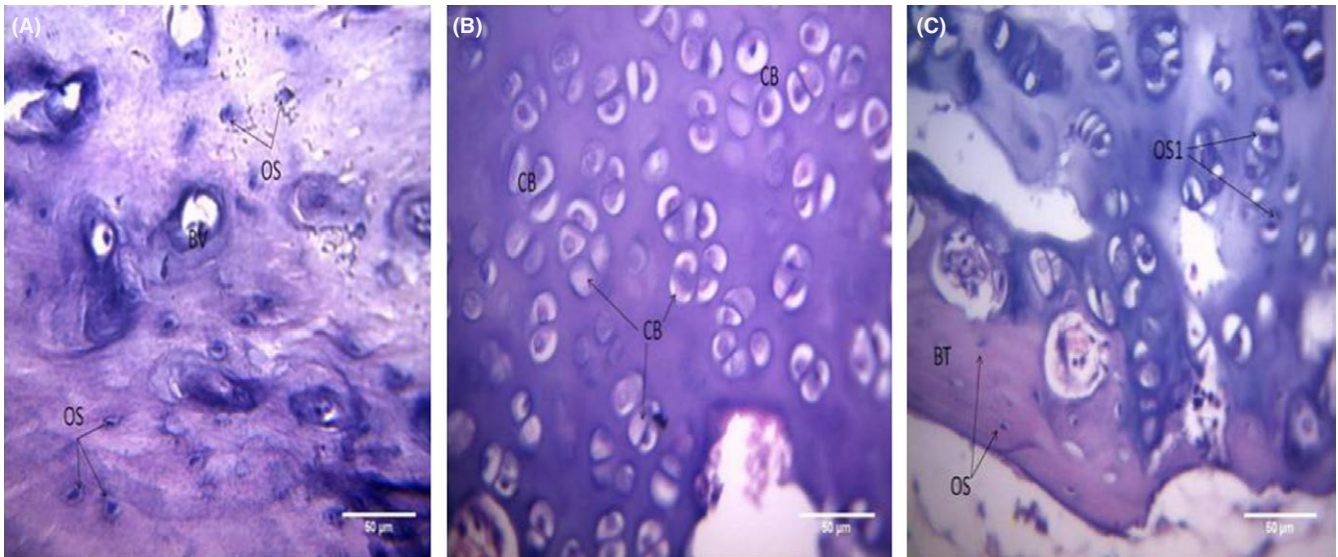
FIGURE 6 Photomicrograph of femur of a Wistar rat at month 3. A, Group treated with CdCl$_2$, showing a considerable number of hypertrophied lacunae enclosing necrotic cells (OS) compared to the D-3-O-methyl-chirioinositol-treated rat (B), showing lacunae that present considerable cartilaginous mode (CB). This is also seen in the control group (C), displaying a normal growth process. The osteocytes labelled OS1 are dividing in the lacuna. Note the blood vessel (BV) in A
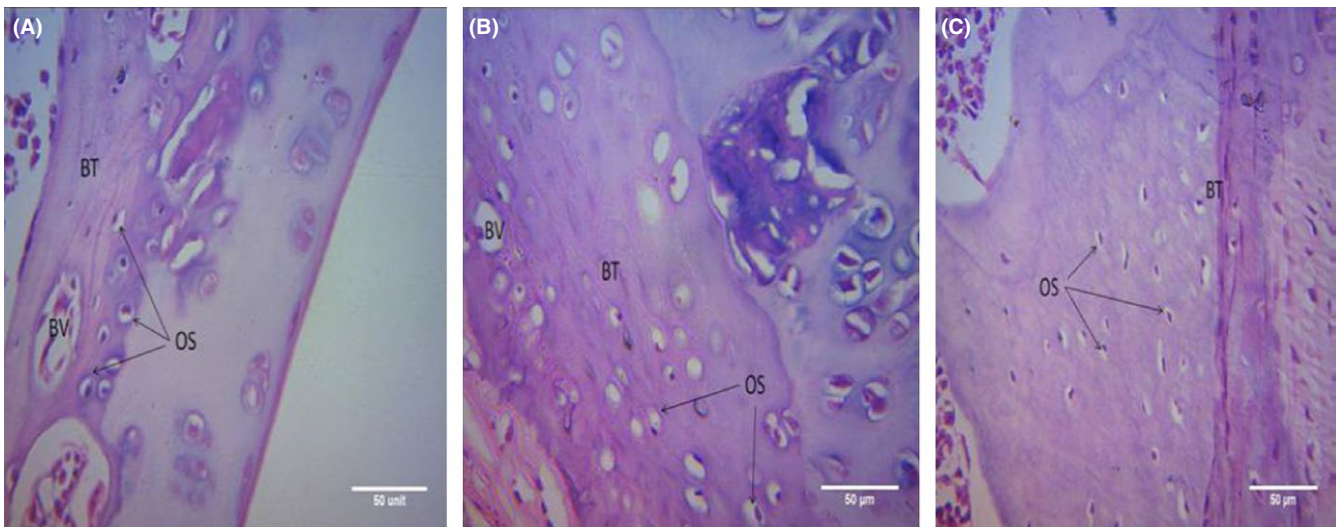

FIGURE 7 Photomicrograph of vertebral disc of a Wistar rat at month 1. A, Group treated with CdCl$_2$. B, Group treated with D-3-O-methyl-chiroinositol. The two treated groups show no visible lesions in the bone trabeculae (BT) and the lacunae, in which the osteocyte (OS) is located. C, Control group. There were no visible lesions in any of the groups in the first month

the experimental groups and time ($F_{2.04,6.121}$ = 2.279; P = 0.182). The tests of within-subjects effects reveal significant variation the in phosphorous concentration between experimental groups in the period of study ($F_{4,12}$ = 9.824; P = 0.001). There were significant differences in magnesium concentration with time ($F_{2,12}$ = 51.257; P < 0.0001) and between the three experimental groups ($F_{4,12}$ = 268.454; P < 0.0001). There were also significant differences in zinc concentration with time ($F_{1.133,6.799}$ = 20.314; P = 0.003) and between the three experimental groups ($F_{2.266,6.799}$ = 9.21; P = 0.011).

Histopathology of the tibial, cervical, and femoral bones of Wistar rats treated with D3O showed bone trabeculae and viable osteocytes.

The CdCl$_2$-treated group showed necrotic and severely hypertrophied osteocytes and there was significant proliferation of blood vessels in the CdCl$_2$-treated group, showing attempts at revascularisation and repair (Figures 4-9). Lacunae counts showed significant variation within groups and across the groups with time (Figure 10).

4 | DISCUSSION

The results of this study demonstrated that cadmium can act directly on bone cells in the bone organ to decrease bone formation
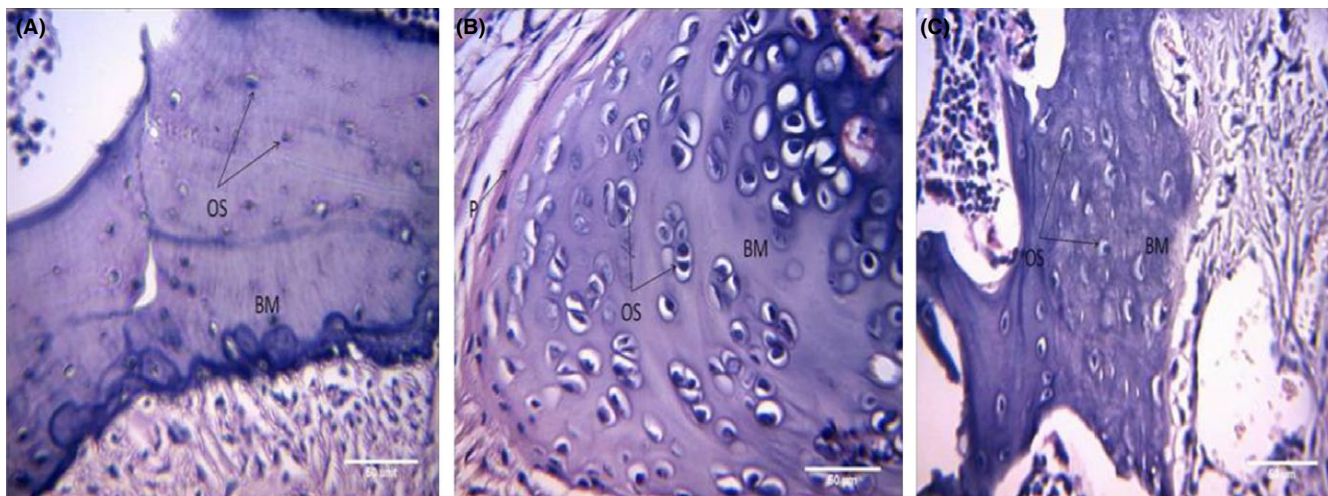
FIGURE 8 Photomicrograph of vertebral disc of a Wistar rat at month 2. A, Group treated with CdCl$_2$ showing hypertrophied osteocytes (OS). B, Group treated with D-3-O-methyl-chiroinositol. There was no visible lesion in the bone matrix (BM), the lacunae in which the osteocytes (OS) are contained and the periosteum (PO). C, Control group. There were no visible lesions in the D-3-O-methyl-chiroinositol-treated group (B) compared to the control group (C)
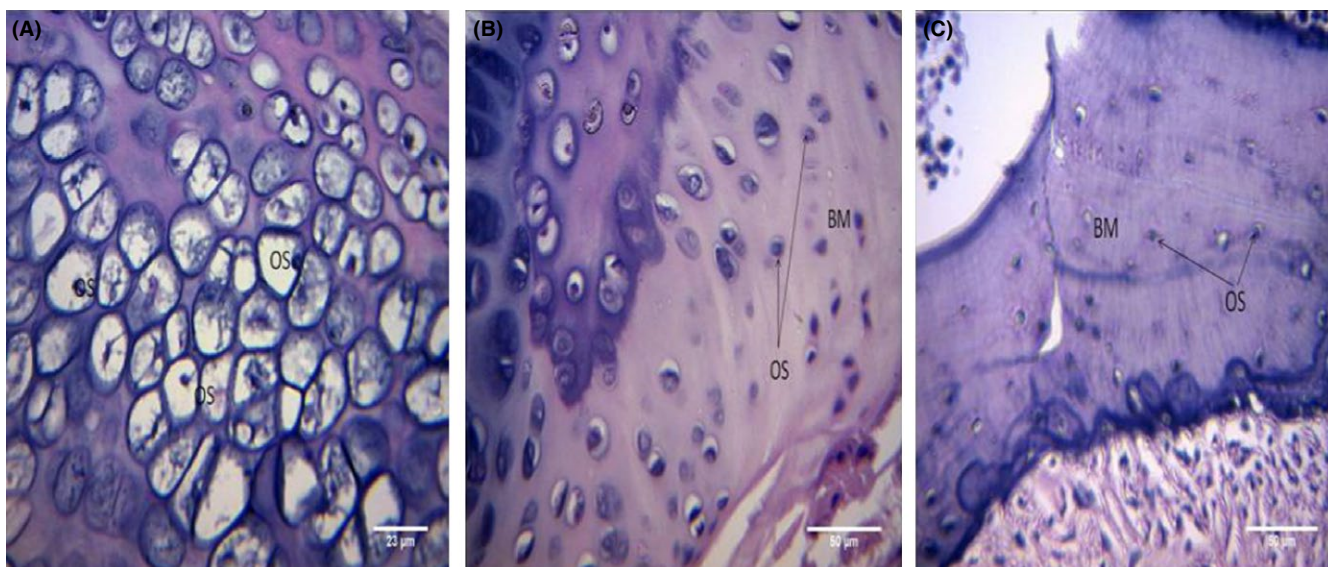

FIGURE 9 Photomicrograph of vertebral disc of a Wistar rat at month 3. A, Group treated with CdCl$_2$, showing mild prominence of the lacunae with osteocytes (OS). B, Group treated with D-3-O-methyl-chiroinositol. C, Control group. There was no visible lesion while in the D-3-O-methyl-chiroinositol-treated group (B) compared to the control group (C)

and increase bone resorption. These results strongly support the hypothesis that cadmium, at low levels of exposure, can act directly on bone cells, in the absence of other effects, to cause osteoporosis. A study reported that even low heavy metal concentrations can increase the risk of osteoporosis.[25] Another study[17] also reported that higher cadmium concentrations led to lower bone densities. However, the mechanism involving the association between heavy metals and bone damage has not been fully explained, although there have been reports that kidney damage is an important pathway for bone loss, and that subsequent calcium malabsorption and iron deficiency in the body increases heavy metal absorption, which, if prolonged, causes proteinuria and consequent bone lesions.[12]

It has, however, been established that cadmium causes osteoclast activation,[26] which results in replacement of the calcium in hydroxyapatite.[25,27] This reduces the strength of the bone by interfering with collagen production in the bone.[28,29] It also increases the urinary calcium excretion,[30,31] thereby leading to osteoporosis.

Mature bone-resorbing osteoclasts are derived from monocyte/macrophage lineage precursors in bone marrow by a process of cellular differentiation called osteoclastogenesis. Osteoclastogenesis is modulated by two key cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL), which are produced by osteoblasts and immune cells. Akesson et al[25] and Beglund[27] reported that calcium
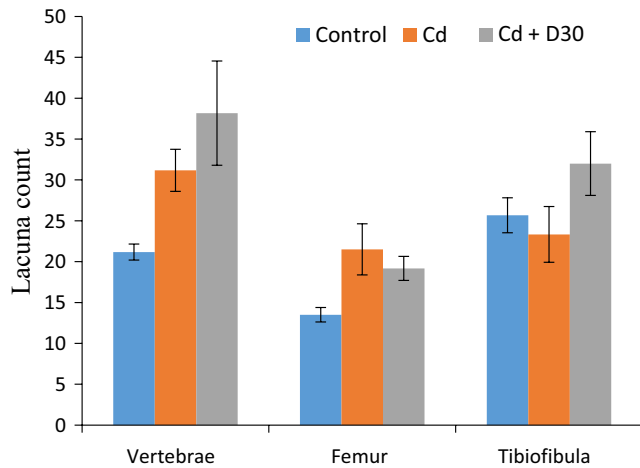
FIGURE 10 Mean changes in lacunae counts of vertebral bone, femur, and tibia of Wistar rats at months 3, 6, and 9, respectively

can be replaced with cadmium in hydroxyapatite to reduce its strength. Cadmium may also interfere with collagen production in the bone[28,29] and increase urinary calcium excretion.[30,31]

Fibroblast growth factor 23 (FGF23) is a bone-derived phosphaturic factor and is known to regulate blood inorganic phosphate (Pi). Cadmium, a toxic transition metal, which is widely used in industry, affects human health through occupational and environmental exposure. Phosphaturia has been documented following cadmium exposure in both human and experimental animals. In this study there were significant changes in serum phosphorus concentration in the treated group compared to the control. In a previous animal study, cadmium administration increased serum FGF23 concentrations. Moreover, medication such as infusion of selected iron-containing compounds increases serum FGF23 concentrations, with patients developing hypophosphatemia. Thus, a relationship between the metal ions (Cd and Fe) and the FGF23 metabolic pathway was proposed.[32]

There was no significant difference in the hydroxylproline concentration across groups studied in this research. Hydroxyproline (OH-Pro) is present in essentially all tissues and all genetic types of collagen. It is derived from the breakdown of collagen. The majority of the breakdown products are reabsorbed by the renal tubules and metabolized in the liver, whereas only about 10% is excreted in the urine. Most are contained in di- and tripeptides.[33,34] The remaining peptides in the urine are approximately 5 kDa in mass. There is a small amount of the free amino acid in urine. OH-Pro can never be reincorporated into newly synthesized collagen,[35] but both collagen synthesis and tissue breakdown contribute to urinary OH-Pro. Colorimetric methods or high-performance liquid chromatography (HPLC) are commonly used to measure urinary OH-Pro. It is historically important as a marker of bone resorption, and is still a good marker when measured correctly. As a matter of fact, studies using calcium kinetics show a better correlation between bone resorption and OH-Pro than some supposedly more specific markers.

Histological studies revealed a high level of bone mineralisation in the CdCl$_2$-treated group, indicative of osteoporosis. Histopathology of the tibial, cervical and femoral bones of Wistar rats treated with D-3-O-methyl-chiroinositol showed bone trabeculae and viable

osteocytes. The CdCl$_2$-treated group showed necrotic and severely hypertrophied osteocytes and there was significant proliferation of blood vessels in the CdCl$_2$-treated group, showing an attempt at revascularisation and repair. The photomicrograph of the femur of a Wistar rat treated with D3O at month 2 showed osteocytes in normal lacunae, whereas a considerable number of the lacunae in the bony mass were hypertrophied, enclosing necrotic cells in the CdCl$_2$-treated group. The photomicrograph of the femur of a Wistar rat at month 3 treated with D3O showed functional lacunae that presented considerable cartilaginous mode and a high level of osteogenesis and repair. There were, however, a considerable number of hypertrophied lacunae enclosing necrotic cells in the CdCl$_2$-treated group compared to the control.

From the results of the study, it was evident that D3O administration led to a partial repair of the osteoporotic damage induced by chronic cadmium exposure in the Wistar rats. Although dual X-ray absorptiometry (DXA) is considered a sensitive assessment of bone mineral density (BMD), it cannot distinguish calcium from Cd. Thus, DXA BMD measures may be upwardly biased due to not differentiating Cd (and other heavy metals) from actual bone mineral content.[36] Downregulation of NF-κB may have been a major mechanism by which D3O regulated osteoclastogenesis and reduced osteoporosis in this research. It is therefore suggested that further studies be carried out to determine the mechanism of action of D3O on osteoclast regulation and osteoporosis. Bone-related diseases and inflammation are major health challenges in our society, especially among the aging population. The peoples of West Africa, especially the eastern regions of Nigeria use a concoction of the stem bark of *Piliostigma thonningiii*, from which D-3-O-methyl-chiroinositol can be isolated, in the treatment of inflammation, bone-related diseases and rheumatoid arthritis. The results of this study suggest that D-3-O-methyl-chiroinositol ameliorated chronic cadmium chloride-induced osteoporosis in male Wistar rats.

5 | CONCLUSION

The authors conclude that D-3-O-methyl-chiroinositol extract from *Piliostigma thionningii* stem bark ameliorated cadmium chloride-induced osteoporosis in male Wistar rats, possibly by downregulation of osteoclastogenesis.

CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

EAU and CON designed the study, EAU, CON, CNA did the experimental research, EAU, CON and TON wrote the initial manuscript,

EAO and OBO did the data analysis and interpretations and EAU, CNA and OKE performed the histological study. All authors proof-read every submission.

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