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Extracts of Eastern Nigeria Mistletoe, *Tapinanthus globiferus* (A. Rich.) Tiegh. Modulate Dexamethasone-induced Insulin Resistance and Exhibit Potent osteogenic Activity in Animal Experimental Model

Omeje Edwin Ogechukwu[1,2,3*], Mohd Parvez Khan[3], Osadebe Patience Ogoamaka[1], Okoye Theophine Chinwuba[4], Ugwoke Christopher Emeka[5], Onugwu Lawrence Ekene[1], Deepshika Tewari[3], Rakesh Maurya[3] and Naibedya Chattopadhyay[3]

[1]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Nigeria, 41001, Nsukka, Nigeria.
[2]Division of Medicinal and Process Chemistry, CSIR-Central Drug Research Institute, Chattar Manzil, P.O.Box 173, 226001, Lucknow, India.
[3]Division of Endocrinology, CSIR-Central Drug Research Institute, Chattar Manzil, P.O.Box 173, 226001, Lucknow, India.
[4]Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, 41001, Nsukka, Nigeria.
[5]Department of Pharmacognosy and Enviromental Medicine, Faculty of Pharmaceutical Sciences, University of Nigeria, 41001, Nsukka, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author OEO was largely responsible for the design, collection of plant materials and execution of the experiment as well as writing of the initial draft of manuscript. Author MPK contributed largely in the design and execution of the research work. Authors NC and OPO were responsible for the overall supervision of the work and editing of manuscript. Author RM assisted greatly with the extraction process. Authors OTC and OLE assisted with the development of the manuscript. Author DT participated actively in the research design and statistical analysis while author UCE assisted with the plant identification alongside Mr. A. O. Ozioko and also formed part of the editorial team. All authors read and approved the final manuscript.

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**Corresponding author: Email: edwin.omeje@unn.edu.ng;*

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ABSTRACT

Ethnopharmacological Relevance: Scientific evidence for the traditional use of eastern Nigeria mistletoe as an anti-diabetic and anti-osteoporotic agent has been documented. In our continued efforts to provide further evidence towards eventual approval of the medicinal usage of the eastern Nigeria mistletoes, the present study was undertaken to evaluate its effects on steroid induced insulin-resistance and *in-vivo* bone health parameters in rodent model.

Materials and Methods: Twelve-weeks (12) old Sprague-Dawley (SD) rats of both sexes and weighing 200±20 g were administered with dexamethasone (200 $mgkg^{-1}$) for minimum of 14 days and with or without 100-400 $mgkg^{-1}$ of crude aqueous extract of mistletoe harvested from *Kola acuminata*, in this paper referred to as Kola-mistletoe (KM). The basal glucose levels were established and animals were later exposed to oral glucose tolerance test (OGTT). Blood samples were collected from ketamine-anesthetized animals via cardiac puncture for measurement of biochemical parameters. The femur and vertebrae bones were neatly excised and stored in 70% isopropanol at 4°C until further analysis. µCT determination of excised bones was carried out using the Sky Scan 1076 µCT scanner (Aartselaar, Belgium) as described in our previously published protocols.

Results: The extracts significantly preserved the animals from the effects of dexamethasone mostly at 400 mg/kg dose in terms of weight loss and blood glycaemia. The extract (either alone or in combination with dexamethasone and within the duration of treatments showed better bone quality (higher BV/TV (Bone volume to Trabecular volume ratio), lower Tb.Sp (Trabecular separation) and SMI (Structure model index) indices at the dose of 400 mg/kg compared with vehicle and metformin groups.

Conclusion: The present data show that the Nigeria mistletoes are potent in reversing the adverse effects of prolonged exposure to dexamethasone in rodents and evidently protected the bones from impairment.

Keywords: Dexamethasone; insulin-resistance; Eastern Nigeria mistletoe; osteogenic; Loranthus micranthus; Tapinanthus globiferus.

1. INTRODUCTION

Insulin-resistance induced by prolonged glucocorticoids therapy is an essential metabolic challenge leading to impaired glucose metabolism, diabetes, obesity, osteonecrosis and ultimately osteoporosis usually referred to medically, as glucocorticoid-induced osteoporosis [1,2]. Glucocorticoids are, unavoidably, widely prescribed in several cases of inflammation including but not limited to rheumatoid arthritis, asthma, systemic lupus erythematosus, cancer and organ transplantation [3,4]. Notably, the adverse effect of glucocorticoids on bone growth and metabolism has been recognized for more than six decades [5,6]. This bone effect is characterized by decreased bone formation and *in situ* apoptosis (osteonecrosis) of isolated segments particularly at the trabecular-rich femoral head [6]. Although, postmenopausal osteoporosis, characterized by rapid loss of mineralized bone tissue, disruption of trabecular architecture of the bone and changes in the crystalline properties of mineral deposits with concomitant effect of structural failure (fracture of sites rich in cancellous bone) affects both female and male, the incidence is much higher in women [7]. This obviously, is attributed to the dramatic estrogen withdrawal associated with menopause. Besides this major causative factor promoting osteoporosis, there are avalanche of evidence indicating that most often, more women than men use glucocorticoids for several indirect medical reasons thereby promoting a faster onset of osteoporosis beyond menopause. Hormones such as catecholamine, glucagon, cortisol and thyroxin, either through directly or their influence on other hormones, affect carbohydrate metabolism to elevate blood glucose level leading to insulin resistance [8].

Insulin-resistance has been shown to be present in conditions like type-II diabetes, obesity and dyslipidemia. Thus, interventions to decrease insulin-resistance may postpone the development of type-II diabetes and its complications [9]. Glucocorticoids in excess inhibit insulin secretion from pancreatic beta-cells, decrease glucose utilization, and stimulate glucagon secretion, lipolysis, proteolysis, and hepatic glucose production. Glucocorticoids can modulate the insulin action at both binding sites and post binding sites and cause decreased glucose utilization in muscles. Glucocorticoids also cause insulin resistance by decreasing hepatic glucose utilization, decreasing glycogen synthesis. Free fatty acids may be elevated in insulin resistance because of impaired insulin-dependent down-regulation of lipolysis, hence leading to increase in triglyceride levels in muscles as well as other tissues presumably because of excess of circulating free fatty acids which are then deposited in these organs. The triglycerides are reported to be potent inhibitors of insulin signaling cascade and result in acquired insulin resistance state [10].

The above facts taken together, glucocorticoid-associated insulin resistance is strongly tied with impairment of glucose and bone metabolism. Experimentally, different doses (low to high) of dexamethasone, a common glucocorticoid, have been demonstrated to induce insulin- resistance and diverse metabolic effects in rodents [11,12]. Expectedly, therefore, an ideal antidiabetic agent should in addition to its normal glucose regulatory potentials modulate insulin resistance effectively and reverse the adverse osteonecrotic effects of implicated agents especially antidiabetic patients on prolonged glucocorticoid.

Medicinal plants have formed the basis of health care throughout the world since the earliest days of humanity and have remained relevant in both developing and the developed nations of the world for various chemotherapeutic purposes [13]. The use of plant-derived natural compounds as part of herbal preparations for alternate source of medicament continues to play major roles in chemotherapy especially in third world countries [14,15]. Several studies carried out have shown that traditional medicines could provide better glycaemic control than currently used conventional drugs [16-19]. In fact, the nearest future will witness a fast growing dependence on herbal medicines for the health needs of world's populations, especially with the avalanche of advancing research in science and technology-biotechnology, molecular biology, plant genomics, and fairly recently, metabolomics. In the different geographical regions of Nigeria, several medicinal plants have been evaluated and found to have potent antidiabetic activities. Notable amongst these antidiabetic plants is the Nigeria species of mistletoe [20,21]. We further evaluated the antidiabetic properties of the eastern Nigeria mistletoes and results obtained led credence to the strong claim and ethnomedicinal uses especially as an antidiabetic [19,22]. Recently too and in specific terms, Ogbonnia et al. [23], reported the potent antihyperglycaemic and andtihyperlipidaemic activities of the *Tapinanthus* globiferus, the mistletoe specie commonly available in Nigeria and notably in south eastern region. Mistletoe, a semi-parasitic plant growing on different trees and shrub occurs in all continents of the world and its biological activities have been shown to be influenced mostly by the topography and climate, host tree and season of harvest [21]. In our recent paper, we documented the various traditional uses of the eastern Nigeria mistletoes and isolated some compounds from the eastern Nigeria mistletoes which exhibited potent osteogenic potentials *in vitro* [24]. Currently, mistletoe leaves in different processed forms are not only used traditionally for the numerous clinical conditions, but are also sold in most registered pharmaceutical outlets in Nigeria. This calls for a comprehensive evaluation of the total health benefits of this plant. Our interest to evaluate the effects of mistletoe extracts on dexamethasone-induced insulin resistance and on bone cell parameters was stirred by the strong scientific evidence supporting its potent antidiabetic action [19,21,22] and the folkloric claim of its ability to control postmenopausal syndrome [25]. We hypothesize that mistletoe extract, a known potent antidiabetic agent will reverse insulin-resistance with concomitant mitigation of the deterioration of bone functions occasioned by long therapy with dexamethasone. The present study is therefore, an attempt to test this plausible hypothesis.

2. EXPERIMENTAL

2.1 Materials and Methods

2.1.1 Collection of plant materials

The leaves and twigs of the eastern Nigeria mistletoes; *Tapinanthus globiferus* (A. Rich.) Tiegh. formerly known as *Loranthus micranthus*

Linn. (Loranthaceae) parasitic on the host tree *Kola acuminata* (Linn.), was collected in October 2011, from different locations in Nsukka LGA, Enugu state. The leaves were identified and certified by Mr. A. O. Ozioko, a taxonomist of the Bioresources Development and Conservation Programme, Nsukka, Enugu state. Voucher specimens were kept at the centre with the number BDC-1021-011 for reference purposes and in accordance with rules of the University of Nigeria regarding ethics and preservation of biodiversity.

2.1.2 Reagents and chemicals

All fine chemicals Sigma Aldrich (St. Louis, MO) while methanol was obtained from BDH Ltd., (Poole, England), distilled water. (Science Laboratory Technology Unit, University of Nigeria, Nsukka)

2.1.3 Animals used

All animal experimental procedures were prior approved (Institutional Animal Ethics Committee approval number (CDRI/IAEC/2012/17) and conducted as per the guide lines laid by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA 34/199). Sprague-Dawley (SD) rats (10-12 weeks old; 200±20 g each) of both sexes were obtained from the National Laboratory Animal Centre, CSIR-CDRI. Animals were kept in a 12 h light-dark cycle, with controlled temperature (22-24°C) and humidity (50-60%). Standard rodent chow diet and water were provided *ad libitum*.

2.2 Methods

2.2.1 Preparation of crude aqueous extract

The leaves of *Tapinanthus globiferus* (A. Rich.) Tiegh. parasitic on the selected host tree (*Kola acuminata*) were cleansed and dried under shade for 8 days. They were pulverized in mechanized laboratory grinder to fine powder. A total of 2.0 kg of the powdered plant material was macerated repeatedly with distill water (total volume; 6 L) The resulting aqueous extract were lyophilized under vacuum afford dry powdered extract which was weighed and its percentage yield was calculated. The dry extracts were placed in amber-coloured glass bottles and stored in a refrigerator until use. The yield obtained was found to be 24.1%. The extract was completely solubilized in normal saline and diluted accordingly to a chosen regimen.

2.2.2 *In vivo* studies

Seventy-two adult SD rats (60 females and 12 males) were randomly divided into 8 equal groups of 6 animals each and another 2 groups (n=12) as follows: Vehicle group (gum acacia in distilled water p.o.), 100 mg/kg (p.o.) of extract group; 200 mg/kg (p.o.) of extract group; 400 mg/kg (p.o.) of extract group; 100 mg/kg (p.o.) of extract + 200 µg/kg (i.p.) dexamethasone group; 200 mg/kg (p.o.) of extract + 200 µg/kg (i.p.) dexamethasone group; 400 mg/kg (p.o.) of extract + 200 µg/kg (i.p.) dexamethasone group; Metformin 200 mg/kg (p.o.) + 200 µg/kg (i.p.) dexamethasone group; two dexamethasone groups 200 µg/kg (i.p.; female and male; n=12 each). All animals received the above doses daily for 21 days and were closely monitored. The doses were chosen based on our already established protocols [25-27]. Daily weight and any obvious signs (weakness, lethargy, anorexia etc) of each animal were recorded. The animals were fasted for 24 hours and the blood glucose levels were checked at the onset and termination of the study with the Accu-Check® glucometer instrument. Oral Glucose tolerance test (OGTT) was performed on all groups by loading each animal with an oral glucose dose of 2 g/kg following 16-24 hours of fasting and monitoring the glycaemic levels at intervals of 30 minutes for a total period of 2 hours. Finally, all animals were sacrificed on the last day to collect blood (via cardiac puncture), femur, tibia, L5 vertebrae following anesthesia with ketamine. The blood samples centrifuged (7500 rpm; 4°C) to collect serum which was stored at -20°C prior to analysis. Bone samples were stored in 70% isopropanol at 4°C until further analysis. Total triglycerides and cholesterol content of serum (to assess dyslipidemia). All measurements were carried out in at least 5 replicates.

2.2.3 Assessment of various parameters for excised bones

µCT determination of excised bones was carried out using the Sky Scan 1076 µCT scanner (Aartselaar, Belgium) as described in our previously published protocols [28,29]. Femurs, tibias and L5 vertebrae dissected from the animals after sacrifice were cleaned of soft tissue prior to scanning. The samples were scanned in batches of two at a nominal resolution (pixels) of 18 µm. Reconstruction was carried out using a modified Feldkamp algorithm using the Sky Scan Nrecon software, which facilitates network distributed reconstruction carried out on four

personal computers running simultaneously. The x-ray source was set at 70 kV and 142 mA, with a pixel size of 18 µm. A hundred projections were acquired over an angular range of 180^0. The trabecular bone of femur head and the callus-dense region of L5 vertebrae were selected by drawing ellipsoid contours with the CT analyzer (CTAn, Skyscan). Micro-architectural parameters including Trabecular bone volume (BV), trabecular numbers (Tb.N), trabecular separation (Tb.sp) and structure model index (SMI) were calculated using the 3D-trabecular analysis software of Skyscan [30,29,31]. The bone mineral density (BMD) of the trabecular bone of femur head and the callus-dense region of L5 vertebrae were computed from their binary images derived from reconstructed volume of interest using CTAn software.

2.2.4 Assessment of mechanical strengths of isolated bones

After µCT measurements, femurs were subjected to three point bending with Bone strength tester Model TK 252C (Muromachi Kikai Co. Ltd., Tokyo, Japan), according to our previously published protocols (Gupta et al. [29]; Siddiqui et al. [28]; Verdelis et al. [30]). Biomechanical parameters of femur including maximum power, stiffness and energy were determined. Taking the mean of control values we derived individual control value as a ratio of the mean ×100 and data expressed as percentage change over the mean value of vehicle group for the various biomechanical parameters. This was also carried out for the L5 vertebrae bones following established protocols.

2.3 Statistical Analysis

Data are expressed as mean ± SEM. The data obtained in experiments with multiple treatments were subjected to one-way ANOVA followed by Turkey's test of significance or Newman-Keuls post-hoc multiple comparism test using Prism 3.0 version software. Where necessary, Student's't' test was used to study statistical significance in experiments with only two treatments.

3. RESULTS

3.1 *In vivo* Studies with Dexamethasone Treated SD Rats of Both Sexes

In order to further strengthen the scientific data supporting the potent antidiabetic activity of the eastern Nigeria mistletoe, we studied it effects in SD rats treated concomitantly with standardized dose of the glucocorticoid, dexamethasone. This was to evaluate the potentiality of the extract preparation in modulating insulin resistance viz-a-viz bone health impairment occasioned by the steroid therapy. The mistletoe harvested from *Kola acuminata* was chosen based on our previous reports and the present data. The results from the *in vivo* studies are shown in Fig. 1 and in Tables 1-3. The effect of extract on the growth rate of SD rats administered with daily dose of 200 µg/kg dexamethasone for 16 days (1A): females, (1B) females and males from independent experiments. Rats treated with extracts alone had comparable growth rate to the vehicle and positive (metformin) controls and at the dose of 400 mg/kg, significantly (**p<0.01) reversed severe weight loss caused by dexamethasone administration. 1C: The final (at the end of experiment) fasted plasma glucose level were significantly (***p<0.001, **p<0.01, *p<0.05) higher than the initial (prior to commencement of experiment) in dexamethasone-treated rats when compared to vehicle and positive controls with the 400 mg/kg extract dose being the most potent. 1D: The extract and dexamethasone treated female SD rats showed low sensitivity to OGTT. Evidently, the plasma glycaemic levels of all the animals exposed to 2 g/kg oral glucose normalized within 2 hours. The male SD rats were resistant to OGTT even after 25 days (data not shown). 1E: The extracts either alone or in combination with dexamethasone summarily, did not affect the mean uterine weights of the female animals when compared to vehicle control. Animals with edematous uterus were observed (possibly caused by the significant mineralocorticoid action of dexamethasone) and were as such eliminated from the data. 1F: There was slight but significant (*p<0.05) increase in the mean pancreatic weight of animals at the dose 400 mg/kg extract plus dexamethasone and Metformin groups, and very significant (***p<0.001) gain in mean pancreatic weight in dexamethasone-alone group compared to the vehicle group. All values are expressed as mean ± SEM (n=6). Table 1 shows that the extract, at the tested doses protected the animals from mortality (100% protection) caused by the adverse effect of the steroid on glucose and energy metabolism compared with the female SD rats treated with dexamethasone alone (50% mortality). In addition, the surviving dexamethasone treated animals were generally weaker, lethargic and anorexic when compared to those receiving extracts simultaneously. The

effect of the extract on some serum lipid profile (total triacylglycerides (TG) and cholesterol (TC)) and serum calcium (Ca^{++}) levels of female SD rats is as shown in Table 2. Treatment of animals with dexamethasone significantly (***p<0.001) increased TG levels compared to vehicle group and extract alone (***$^{\$}$p<0.001) treated. The extracts did not cause a significant change in TG (^{a}) when compared to vehicle group. However, extracts at 200 and 400 mg/kg in combination with dexamethasone caused a significant (*$^{\$\$}$p<0.05) reduction in amount of circulating triglycerides in serum compared to dexamethasone group. Similarly, TC values of extract treated rats alone or in combination with dexamethasone did not vary significantly (NS) from that of vehicle group except for the 100 mg/kg (***p<0.001) extract dose. The extracts at all doses significantly (*p<0.05; **p<0.01; ***p<0.001) preserved serum calcium levels compared to vehicle ad metformin controls and restored serum calcium loss induced by dexamethasone treatment.

Table 1. Mortality rate of animals treated with either extracts or dexamethasone and controls

Group (number)	Death count (number)	Mortality
Vehicle (6)	0	0 %
100 mg Extract (6)	0	0 %
200 mg Extract (6)	0	0 %
400 mg Extract (6)	0	0 %
100 mg Extract + Dexamethasone 200 µg/kg (6)	0	0 %
200 mg Extract + Dexamethasone 200 µg/kg (6)	0	0 %
400 mg Extract + Dexamethasone 200 µg/kg (6)	0	0 %
Metformin 200 mg/kg (6)	0	0 %
Dexamethasone 200 µg/kg (12)	6	50 %
Dexamethasone 200 µg/kg (12; Male SD rats)	0	0 %

Treatment with dexamethasone at 200 µg/kg dose induced severe weight loss, lethargy, weakness and mortality mostly in female SD rats within16 days. The male animals were lethargic but no death occurred in the group. Animals treated with extracts alone or in combination with dexamethasone exhibited a dose dependent resistance of majority of these adverse effects and compared with the controls. The male SD rats were able to survive the treatment period with dexamethasone such that there was zero mortality in the group. However, the animals were generally weak, lethargic and lost substantial weight in the course of the experiment

Table 2. Effect of treatment on some serum lipids and calcium levels after 16 days

Groups	TG	TC	TCa
Vehicle	26.73±2.43	44.20±0.93	8.89±.10
Dexa (200 µg/kg)	51.48±4.96***	52.50±5.93***	8.54±0.27
100 mg/kg extract	30.98±2.17***$^{\$, a}$	51.35±3.26***	9.08±0.10***
200 mg/kg extract	29.03±2.68***$^{\$, a}$	34.98±1.14^{NS}	8.99±0.32***
400 mg/kg extract	30.25±1.91***$^{\$, a}$	35.98±1.61^{NS}	9.13±0.14***
100 mg/kg extract + Dexa	48.35±2.87	37.48±2.09^{NS}	8.72±.10*
200 mg/kg extract + Dexa	40.53±0.48*$^{\$\$}$	36.85±2.13^{NS}	8.84±0.18**
400 mg/kg extract + Dexa	39.40±0.47*$^{\$\$}$	40.50±3.08^{NS}	8.78±0.10*
Metformin 200 mg/kg + Dexa	52.77±2.86	53.47±7.25	8.98±0.14***

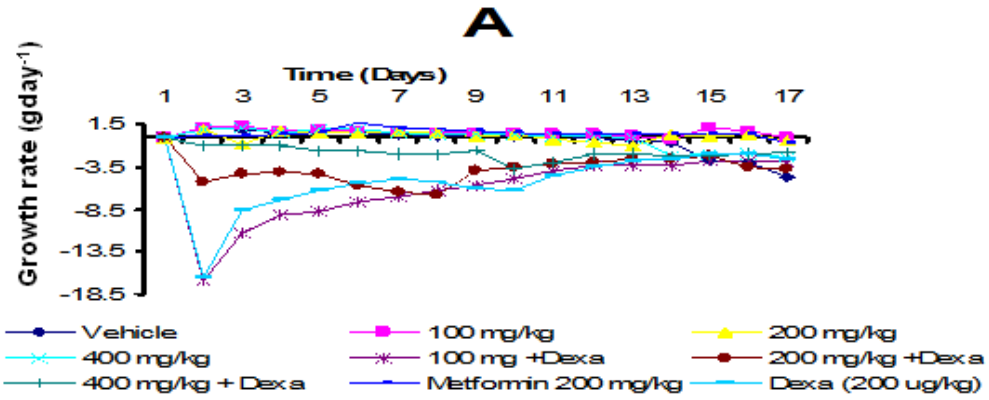
Key: TG (Total triglycerides); TC (Total Cholesterol); TCa (Total calcium)

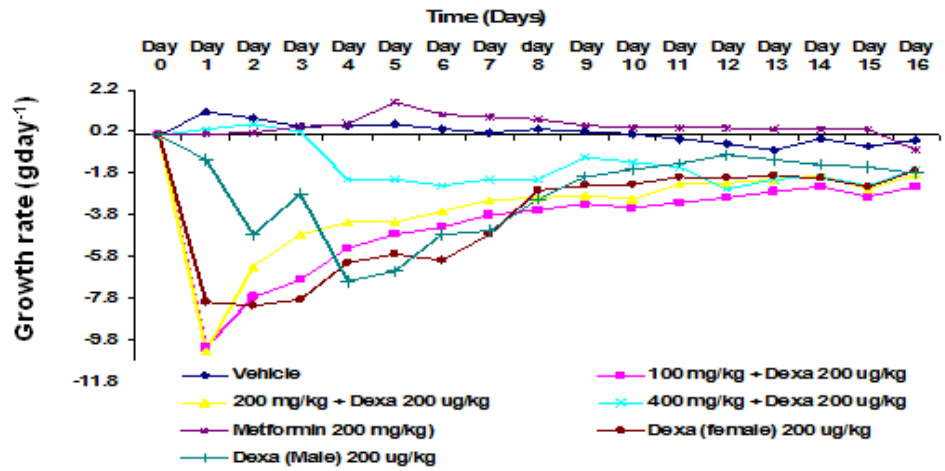
*Values are expressed as mean ± SEM (n=6ix rats/group). One-way ANOVA was used to compare group means. Treatment of animals with dexamethasone significantly (***p<0.001) increased TG levels compared to vehicle group and extract alone (***$^{\$}$p<0.001) treated. The extracts did not cause a significant change in TG (^{a}) when compared to vehicle group. However, extracts at 200 and 400 mg/kg in combination with dexamethasone caused a significant (*$^{\$\$}$p<0.05) reduction in amount of circulating triglycerides in serum compared to dexamethasone group. Similarly, TC values of extract treated rats alone or in combination with dexamethasone did not vary significantly (NS) from that of vehicle group except for the 100 mg/kg (***p<0.001) extract dose. The extracts at all doses significantly (*p<0.05; **p<0.01; ***p<0.001) preserved serum calcium levels compared to vehicle ad Metformin controls and restored serum calcium loss induced by dexamethasone treatment.*

Table 3. Effect of extract on the bone mineral density (BMD) of SD rats exposed to prolonged dexamethasone treatment for a period of 16 days

Groups	BMD (%)	
	Trabecular bone	L5 vertebral bone
Vehicle	1.6486 ± 0.0003	1.6060 ± 0.0004
Dexamethasone (200 µg/kg)	1.5235 ± 0.0003	1.5160 ± 0.0004
Metformin 200 mg/kg + Dexamethasone	1.6716 ± 0.0002***a	1.6951 ± 0.0003***a
Extract 100 mg/kg	1.5918 ± 0.0002*b	1.6736 ± 0.0003***b
Extract 100 mg/kg + Dexamethasone	1.6974 ± 0.0002	1.7878 ± 0.0003***
Extract 200 mg/kg	1.6005 ± 0.0002*	1.6335 ± 0.0003**
Extract 200 mg/kg + Dexamethasone	1.6690 ± 0.0002***	1.7117 ± 0.0003***
Extract 400 mg/kg	1.6221 ± 0.0003	1.6999 + 0.0002**
Extract 400 mg/kg + Dexamethasone	1.6662 ± 0.0002**	1.7532 ± 0.0002***

*Mean BMD ± SEM values were computed electronically from binary images of reconstructed volume of interest (VOI) of trabecular and vertebral bone using the CTAn.exe software of the µCT Skyscan equipment. The extract at all doses either alone or in combination with dexamethasone significantly (***p<0.001; **p<0.01; *p<0.05) enhanced BMD in proportion comparable to the positive control, Metformin and very high when compared to untreated vehicle. ***[a](compared to vehicle group); ***[b], *[b](compared to dexamethasone group). Mistletoe extracts therefore enhanced and preserved bone compactness (quality) of SD rats treated alone with extracts or in combination with 200 µg/kg dexamethasone*





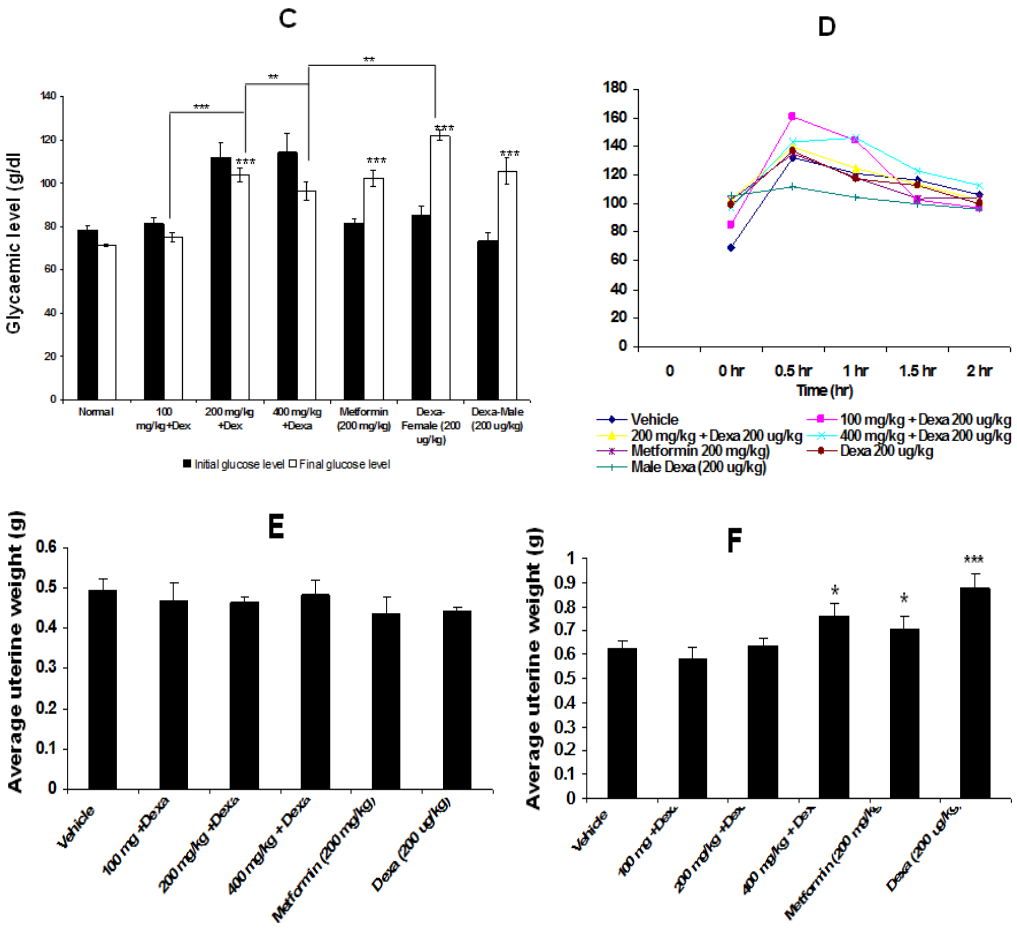
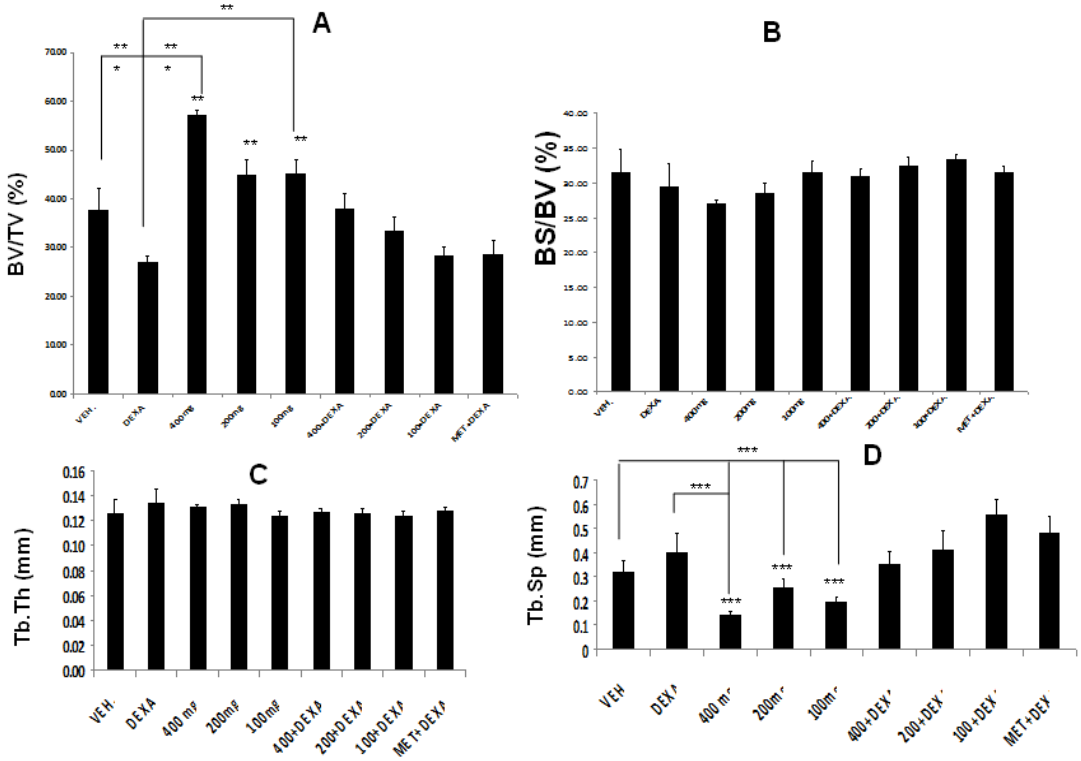


Fig. 1. Effect of extract from mistletoe parasitic on Kola acuminata (KM) on the growth rate of SD rats administered with daily dose of 200 µg/kg dexamethasone for 16 days (1A): females, (1B) females and males from an independent experiment. Rats treated with extracts alone had comparable growth rate to the vehicle and positive (Metformin) controls and at the dose of 400 mg/kg, significantly (p<0.01) reversed severe weight loss caused by dexamethasone administration. 1C: The final (at the end of experiment) fasted plasma glucose level were significantly (***p<0.001, **p<0.01, *p<0.05) higher than the initial (prior to commencement of experiment) in dexamethasone-treated rats when compared to vehicle and positive controls with the 400 mg/kg extract dose being the most potent. 1D: The extract and dexamethasone treated female SD rats showed low sensitivity to OGTT. Evidently, the plasma glycaemic levels of all the animals exposed to 2 g/kg oral glucose normalized within 2 hours. The male SD rats were resistant to OGTT even after 25 days (data not shown). 1E: The extracts either alone or in combination with dexamethasone did not affect the mean uterine weights of the female animals when compared to vehicle control. 1F: There was slight but significant (*p<0.05) increase in the mean pancreatic weight of animals at the dose 400 mg/kg extract +dexamethasone and Metformin groups, and very significant (***p<0.001) gain in mean pancreatic weight in dexamethasone-alone group compared to the vehicle group. All values are expressed as mean ± SEM (n=6)**

3.2 Assessment of Various Parameters for Excised Bones

µCT determination of excised bones was carried out using the Sky Scan 1076 µCT scanner. The result of derived parameters for trabecular and vertebral bones of the animals are shown in Figs. 2A-H and 3A-I. The effect of KM extract on the micro-architectural parameters of trabecular region of SD rats exposed to 16 days dosing with

dexamethasone (200 $\mu gkg^{-1}day^{-1}$). (A) µCT analysis showing BV/TV, bone volume/tissue volume (%); (B) BS/BV, Bone surface/ Tissue volume; (C) Tb.Th (mm), trabecular (strut) thickness; (D) Tb.Sp., Trabecular (strut) separation (mm); (E) Tb.N., Strut number (1/mm); (F) Tb.Pf., Trabecular pattern factor, (G) Con.D., Connectivity density, and (H) SMI, Structure model index. The extract (either alone or in combination with dexamethasone) and within the duration of treatments showed better bone quality (higher BV/TV, lower Tb.Sp and SMI) parameters indices significantly (***p<0.001; **p<0.01;*p<0.05) at the dose of 400 mg/kg compared with vehicle and metformin groups. The large negative SMI values indicate more plates than rods in new bone formation improving the overall compactness and quality of the trabecular bone region. All values are expressed as mean ± SEM (n=x rats/group). Fig.3: Effect of KM extract on the microarchitectural parameters (A-I) of the load bearing L5 vertebrae trabecular region of SD rats exposed to 16 days dosing with dexamethasone (200 µg.kg-1day-1). The extract similarly (either alone or in combination with dexamethasone and within the duration of treatments showed better bone quality (higher BV/TV, lower Tb.Sp, lower SMI etc) parameters indices significantly (***p<0.001; **p<0.01;*p<0.05) at the dose of 400 mg/kg compared with vehicle and metformin groups. Again, the large negative SMI values indicate more plates than rods in new bone formation improving the overall compactness and quality of the L5 vertebrae. The 100 mg dose of extract failed to lower the SMI in the presence of dexamethasone. All values are expressed as mean ± SEM (n=x rats/group). The computed bone mineral density (BMD) is depicted in Table 3. Mean BMD ± SEM values were computed electronically from binary images of reconstructed volume of interest (VOI) of trabecular and vertebral bone using the CTAn.exe software of the µCT Skyscan equipment. The extract at all doses either alone or in combination with dexamethasone significantly (***p<0.001; **p<0.01; *p<0.05) enhanced BMD in proportion comparable to the positive control, Metformin and very high when compared to untreated vehicle. ***[a](compared to vehicle group); ***[b],*[b](compared to dexamethasone group). Mistletoe extracts therefore enhanced and preserved bone compactness (quality) of SD rats treated alone with extracts or in combination with 200 µg/kg dexamethasone.



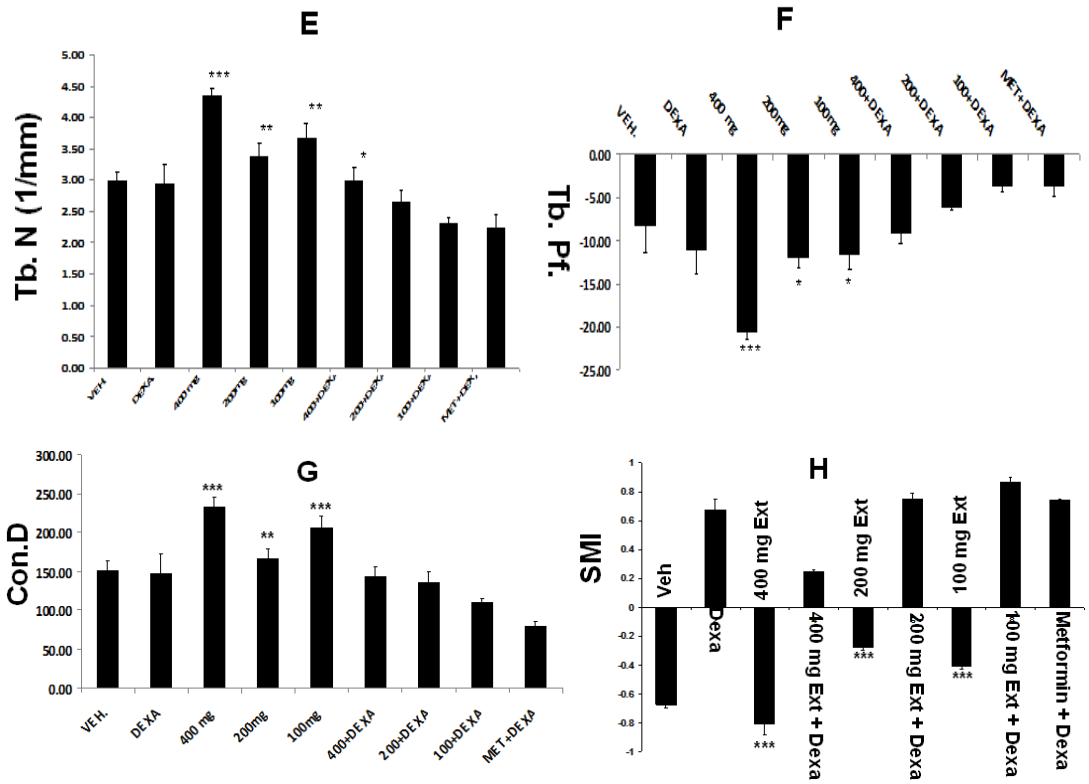
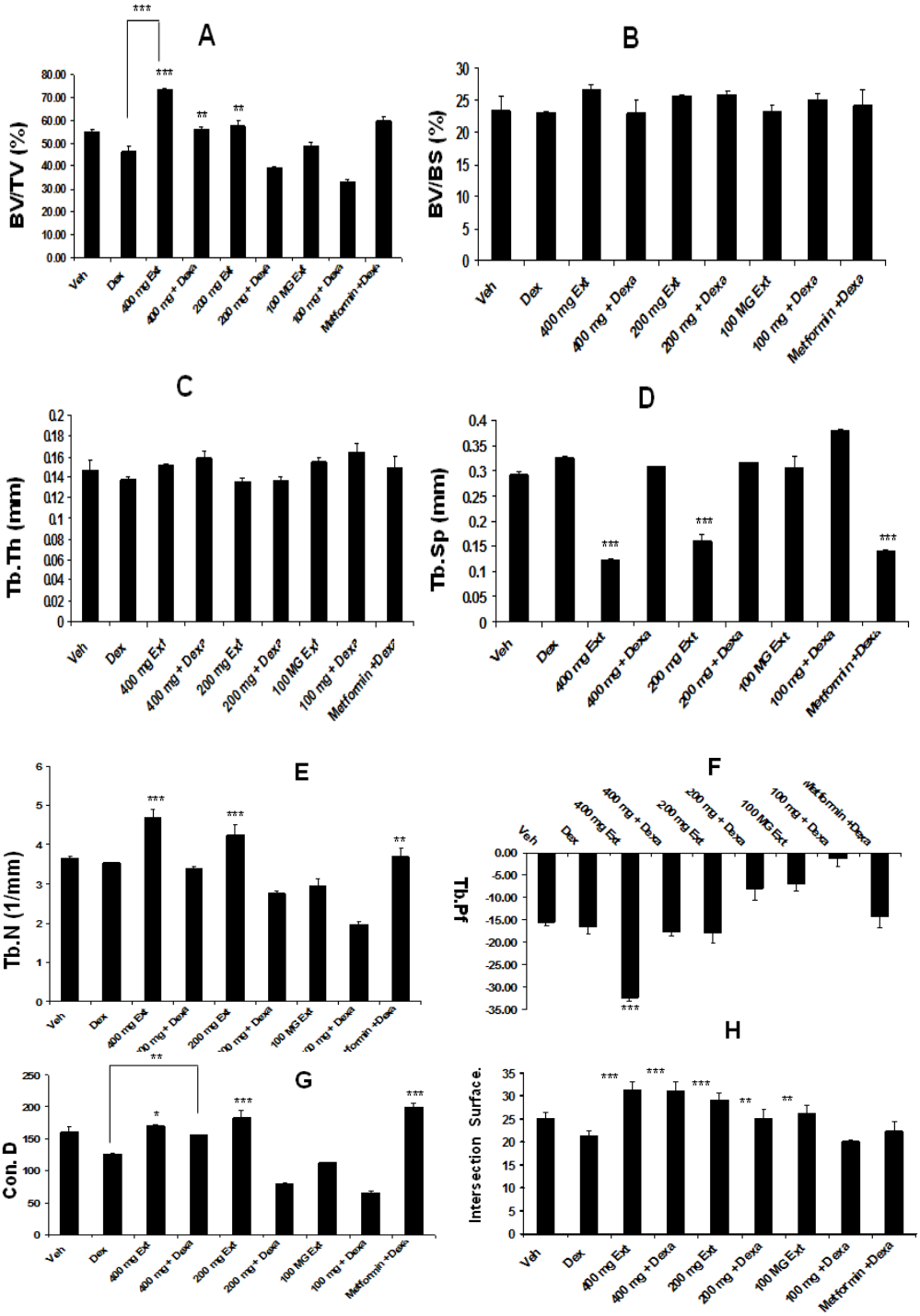


Fig. 2. Effect of treatment with KM extracts on the microarchitectural parameters of trabecular region of SD rats exposed to 16 days dosing with dexamethasone (200 µgkg^{-1}day^{-1}). (A) µCT analysis showing BV/TV, bone volume/tissue volume (%); (B) BS/BV, Bone surface/ Tissue volume; (C) Tb.Th (mm), trabecular (strut) thickness; (D) Tb.Sp. trabecular (strut) separation (mm); (E) Tb.N., Strut number (1/mm); (F) Tb.Pf., Trabecular pattern factor, (G) Con.D., Connectivity density, and (H) SMI, Structure model index. The extract (either alone or in combination with dexamethasone ad within the duration of treatments showed better bone quality (higher BV/TV, lower Tb.Sp and SMI) parameters indices significantly (*p<0.001; **p<0.01;*p<0.05) at the dose of 400 mg/kg compared with vehicle and Metformin groups. All values are expressed as mean ± SEM (n=x rats/group)**

4. DISCUSSION

We recently established the effects of the standardized extracts of eastern Nigeria mistletoe harvested from three host trees (*Kola acuminata, Citrus spp, Garcinia kola*) on the cell viability and ALP activity using cultured rat calvariae osteoblasts [24]. Our findings showed that there was no cytotoxicity at all tested doses (0.2-3.2 µg/ml) compared to control (NS) and at 0.2 µg/ml dose of *Kola acuminata* derived mistletoe extract, there was a significant (**p<0.01) proliferation of the cells. Similarly, ALP activity (a known bone growth biomarker), of treated rat calvariae cells was significantly (*p<0.05, **p<0.01, ***p<0.001) enhanced by all extracts at dose rage of 0.2-1.6 µg/ml compared the vehicle and positive control, BMP2 (100 ng/ml). The doses of the extracts (100-400 mg/kg) and the estimated EC_{50} of the crude extracts were also low which further leads credence to the overall safety of the crude plant extracts. These doses used in the study were extremely low compared to already established LD_{50} of mistletoe extracts in mice (LD_{50} of *Kola acuminata* derived mistletoe extract> 10,000 mg/kg; and an average LD_{50} value > 7500 mg/kg from five different host trees [25]. In addition, some compounds isolated from the leaves of mistletoe demonstrated potent proliferation of C57BL/6 mice splenocytes and significant immunomodulatory potentials [32,33]. These data, taken together imply that the extracts are safe to the bone forming cells and enhance the activity of bone growth biomarker, ALP (positive osteogenic potential). In our continued efforts to

completely characterize the biological effects of local mistletoes used in traditional medical practice of eastern Nigeria, we set to study further activities of the extract in relation to its already established anti-diabetic properties. In the present work, our aim was to investigate the effect of *Kola acuminata*-derived mistletoe extract (KM) on SD rats exposed to prolonged high dose of dexamethasone. In addition, we studied the micro-architectures of designate bones from the treated animals and controls. As have been previously reported [4-6], the



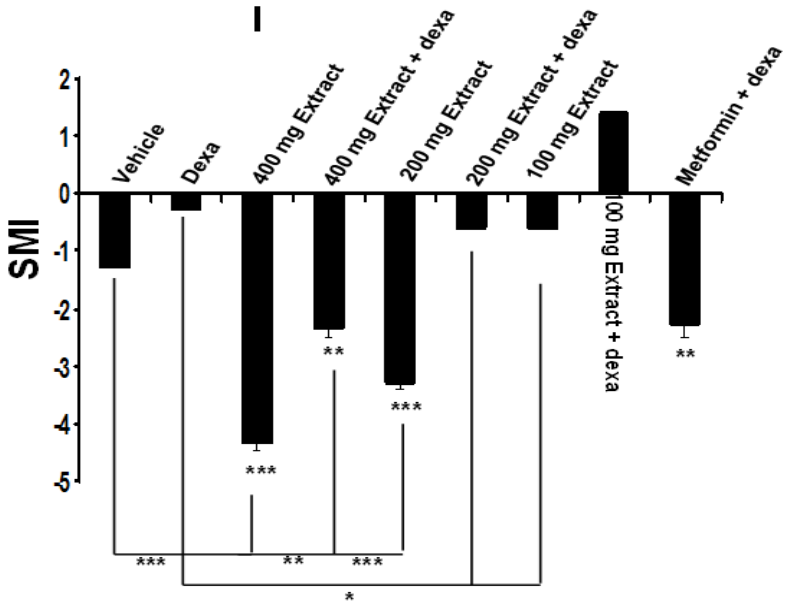


Fig. 3. Effect of treatment with KM extract on the microarchitectural parameters (A-I) of the load bearing L5 vertebrae trabecular region of SD rats exposed to 16 days dosing with dexamethasone (200 µg.kg-1day-1). The extract similarly (either alone or in combination with dexamethasone and within the duration of treatments showed better bone quality (higher BV/TV, lower Tb.Sp, lower SMI etc) parameters indices significantly (*p<0.001; **p<0.01;*p<0.05) at the dose of 400 mg/kg compared with vehicle and Metformin groups. the large negative SMI values indicate more plates than rods in new bone formation improving the overall compactness and quality of the L5 vertebrae. the 100 mg dose of extract failed to lower the SMI I the presence of dexamethasone. All values are expressed as mean ± SEM (n=x rats/group)**

adverse effect of glucocorticoids on bone growth and metabolism is characterized by decreased bone formation and *in situ* apoptosis (osteonecrosis) of isolated segments particularly at the trabecular-rich femoral head [6]. It is expected that the effects of osteogenic extracts or compounds will be manifested more in the female animals because of the interplay between oestrogen and bone metabolism *vi-a-viz,* diabetic syndrome. In the present work, the extract had no significant estrogenic activity which resulted in the non-differentiable action on both sexes in terms of growth rate. More obviously, intake of exogenous steroids like dexamethasone precipitates type-II diabetes which manifests drastic weight loss and increased adipogenesis due to altered glucose metabolism. This results in increased circulating cholesterols, glycaemia and other triglycerides with attendant clinical implications, often insulin resistance. The extract showed a dose dependent reversal effect on weight loss, high glycaemia, high cholesterol and triglycerides in comparison to control groups. Furthermore, the extract at 400 mg/kg body weight dosage was able to protect the loss of calcium ion in the animals dosed with dexamethasone compared with controls. The significance of these findings is that all the effects associated with steroid-induced insulin resistance were either reversed or ameliorated reasonably. This implies that extract of the eastern Nigeria mistletoe used in this study (KM) has added potentials that address the complications of diabetes and insulin resistance. Furthermore, the extract similarly (either alone or in combination with dexamethasone and within the duration of treatments showed better bone quality (higher BV/TV, lower Tb.Sp, lower SMI etc) parameters indices significantly at the dose of 400 mg/kg compared with vehicle and metformin groups. Again, the large negative SMI values indicate more plates than rods in new bone formation improving the overall compactness and quality of the L5 or the load-bearing vertebrae. The 100 mg dose of extract failed to lower the SMI in the presence of dexamethasone. The extract at all doses either alone or in combination with dexamethasone

significantly enhanced the bone mineral density (BMD) in proportion comparable to the positive control, Metformin and very high when compared to untreated vehicle and dexamethasone groups. Mistletoe extracts therefore enhanced and preserved bone compactness (quality) of SD rats treated alone with extracts or in combination with 200 µg/kg dexamethasone. This finding is in agreement with our recent report describing the presence of osteogenic compounds in eastern Nigeria mistletoes [24]. Previously, the strong anti-diabetic properties of the crude aqueous methanol extract was established [21] and its relationship with the present findings is clinically relevant. In conclusion, extract of eastern Nigeria mistletoes parasitic on *Kola acuminata* could become a safe and cheaper alternative in the treatment and or management of insulin resistance in diabetic therapy, secondary osteoporosis and related bone diseases.

5. CONCLUSION

The present data show that leave extracts of eastern Nigeria mistletoes are potent in reversing the adverse effects of prolonged exposure to dexamethasone in rodents and evidently protected the bones from impairment. These extracts could therefore be further developed to serve as a cheaper alternative in the management of diabetes and or insulin resistance as well as secondary osteoporosis occasioned by steroid therapy.

CONSENT

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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