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Aqueous extract of *Alstonia boonei* De Wild (Apocynaceae) alleviates bone metabolism disorders induced by oxidised palm oil and sucrose supplementation in rats

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ABSTRACT

Background: Poor nutritional status disrupts bone metabolism leading to fractures. This study investigated the effect of the aqueous leaf extract of *Alstonia boonei* on fractured rats fed a diet supplemented with oxidised palm oil and sucrose. **Methods**: The iodine, peroxide and acidity indexes of the oxidised palm oil were determined. Bone damage was induced by feeding rats an oxidised palm oil/sucrose enriched diet for 16 weeks followed by fracture. Fractured animals received distilled water or plant extract (50, 100 or 200 mg/kg) simultaneously with their diet for two weeks. Biochemical parameters including lipid profile, bone markers, oxidative stress and hematological parameters were assessed. X-rays and histology of the femur were performed. **Results**: Oxidised palm oil showed a significant increase in the peroxide and acidity indexes, while the iodine index decreased. Fractured rats fed a diet enriched with oxidised palm oil and sucrose exhibited dyslipidemia, altered haematological parameters and oxidative stress bone ALP activity with poor natural bone formation. Fracture induced a decrease in bone calcium and an increase in bone ALP. *A. boonei* extract improved lipid profile, hematological and oxidative stress parameters. There was a significant increase in femoral calcium and a significant decrease in femoral ALP. In addition, a better organisation of the bone architecture was observed, with a marked effect at a dose of 100 mg/kg. **Conclusion**: These results suggest that *A. boonei* leaf improves bone repair in poor nutritional conditions, thanks to its hypolipidemic and antioxidant properties mediated by the minerals and secondary metabolites contained in the extract.

Keywords: Thermo-oxidised palm oil, Sucrose, Fracture, *Alstonia boonei*, Osteoformation.

INTRODUCTION

A fracture is an injury characterised by a break in the continuity of a bone [1]. It can range from a crack in a non-displaced bone to a fracture that involves multiple fragments [2]. These fractures are accidental bone changes due to trauma or bone fragility. Fragility fractures due to osteoporosis are more common in older people [3]. However, dietary behaviours need to be appropriate throughout life to avoid increasing an individual's risk of a fragility fracture. According to the International Osteoporosis Foundation, 9 million fragility fractures occur every year [3]. These types of fractures result from hormone deficiency and/or malnutrition. Malnutrition is a factor that affects bone growth, bone capital, bone quality and bone remodelling. Poor nutritional status can increase the risk of falls leading to fractures and, consequently, poor bone union [4]. Sucrose and/or lipid supplementation has been shown to be detrimental to bone structure [5]. It should also be remembered that exogenous free radicals and/or their endogenous increase cause an imbalance in the activity of osteoblasts and osteoclasts, increasing the alteration in bone metabolism leading to osteoporosis. [6]. Bone has a natural ability to consolidate, but situations such as malnutrition and insulin resistance [7,8] are situations in which this consolidation is delayed [9]. Our eating habits and sedentary lifestyle contribute to insulin resistance, which is the first stage in the onset of type 2 diabetes and is thought to have a negative influence on bone remodelling, increasing the risk of fracture [10].

To maintain its integrity throughout life, bone undergoes remodelling, during which old bone is replaced by new bone [11]. This process is crucial in the repair of fractures [11]. Treatment varies depending on the nature of the area affected. It is generally based on surgical or non-surgical methods, consisting of repositioning the fractured bones and immobilising the affected limb (plaster cast or orthopaedic material) in order to facilitate bone re-growth [12]. Osteo-inducing drugs such as bone morphogenic proteins (BMPs) and anti-inflammatory drugs [13] are used. In some cases, vitamin D supplements combined with calcium and bone grafts are also necessary [7]. Given the expectations for rapid bone consolidation, these drugs, in addition to their side effects, are not always satisfactory [13], hence the use of alternative treatments such as medicinal plants. In fact, the use of medicinal plants as new sources of

medication is constantly increasing, thanks to their secondary metabolites with biological activities. Some of the natural compounds that have been studied and shown to have osteoinductive effects in normal rats include a 2-quercetin analogue isolated from *Ulmus wallichiana* [14] and *Peperomia pellucida*, [15]. In addition, studies have shown that the combination of oxidised palm oil and sucrose has a detrimental effect on carbohydrate and lipid metabolism [16]. However, given the fragility of the bones of malnourished patients and the healing effects of the aqueous extract of *A. boonei* leaves in traditional medicine [17], we proposed to evaluate the healing effects of the aqueous extract of *A. boonei* leaves in rats receiving an oxidised palm oil and sucrose supplement followed by fracture of the femoral shaft.

MATERIALS AND METHODS

Animals

Animal studies were carried out with the approval of the Cameroon National Ethics Committee (Ref n° Fw-IRb00001954). Healthy male albino Wistar rats aged 4 weeks old and weighing between 40 and 60 g were used. These animals were breaded at the Animal Physiology Laboratory of the University of Yaoundé I where they were housed in cages maintained in a ventilated room at room temperature according to a nychthemeral cycle and had free access to tap water and food.

Plant material

Fresh leaves of *Alstonia boonei* (*A. boonei*) were collected in Yaoundé (Centre Cameroon), more precisely in the Ngoa-ekele district in June 2021. The plant was authenticated at the National Herbarium of Cameroon by comparison with the material of Leevwenberg A.J.M. 9191 recorded in the herbarium's collection as N°43365 SRF/cam.

Preparation of the extract

The leaves of *A. boonei* were collected cut into small pieces and dried in the shade, then ground to obtain a powder. The aqueous extract was prepared according to the traditional healer's recommendations; 200 grams of the powder was boiled in 4L of distilled water for 10 minutes. After cooling, the resulting mixture was filtered through Whatman N°3 paper. The filtrate obtained was oven dried at 45°C to obtain the crude extract.

Preparation of a diet supplemented with thermo-oxidised palm oil and sucrose

To obtain the dietary supplement of 10% thermo-oxidised palm oil (*Elaeis guineensis*), 500 mL of palm oil was blanched for 30 minutes over a domestic gas fire. Cooled for 2 hours and reheated for 10 minutes twice after successive cooling following the modified protocol of Owu *et al.* [18]. Ten grams (10g) of this oil was mixed with 90 g of standard chow diet to give a 10% dietary supplement of oxidised palm oil. To obtain the 10% of sucrose, 10 g of SOSUCAM (Société Sucrière du Cameroun) brand blond sugar was introduced into a graduated cylinder and 100 mL of tap water was added. The sugar was quickly dissolved using a magnetic stirrer.

Determination of some chemical properties of thermo-oxidised palm oil

The chemical quality (peroxide value, iodine value and acid value) of the red palm oil purchased at the Etoudi market was immediately analysed as soon as it arrived at the laboratory. The chemical quality of thermo-oxidised palm oil was also determined.

Iodine index titration

The method used is that of the French Federation for Standardisation [19]. A 100 mL conical flask was filled with: 5 mL of 0.5% lipid solution in CCL4, 20 mL of alcoholic iodine solution and 20 mL of alcoholic $HgCl_2$ solution. The flask was corked and allowed to stand in the dark for 2 hours. 25 mL of KI, 2% were added and vigorously shaken for 2 minutes. Iodine went into solution and gave it characteristic brown colour. 10 mL of distilled water were added and the excess iodine was titrated with sodium thiosulphate, N/50. The sodium thiosulphate was added dropwise until the colour changed from brown to pale yellow. One drop of starch solution was added. If this starch showed its characteristic blue colour indicating the presence of iodine, the addition of thiosulphate was continued drop wise until the colour disappears completely. A control flask was done under the same conditions but the lipid solution in CCL_4 was replaced by CCL_4 solvent. 3 trials were done for each sample. The iodine index (I_i) was calculated as follows:

$$\text{Ii} = \frac{0.127\text{XN}(\text{V2} - \text{V1})}{\text{W}}\ x\ 100$$

Ii: Iodine index, Weight expressed in g, fixed per 100 g of fat; N=1/50=0.02: The normality of sodium thiosulphate; V1= Volume of sodium thiosulphate in mL used to titrate the sample; V2= Volume of sodium thiosulphate in mL used to titrate the control; W: Weight of fat

Peroxide index titration

The method used is that of AFNOR [19]. In a 100 mL Erlenmeyer flask, 1 g of oil was weighed, 10 mL of methylene chloride was added to dissolve the oil. 15 mL of concentrated acetic acid and 1 mL of saturated potassium iodide were added. The tube was stoppered, shaken and left in the dark for 5 minutes. 75 mL of distilled water was added and the tube was shaken vigorously. The iodine released was rapidly titrated with a solution of sodium thiosulphate 0.01 N. Two drops of starch were added to act as a colour indicator. The control tubes were prepared under the same conditions; but the oil solution was replaced by the solvent methylene chloride. Three trials were carried out for each sample. The peroxide index, I_P, was calculated as follows:

$$Ip = 1000 \text{ x N x} \frac{(\text{V1} - \text{V2})}{\text{W}}$$

Ip: Number of active µg of peroxide contained in 1g of fat; N= 0,01: The normality of sodium thiosulphate; V1= Volume of sodium thiosulphate in mL used to titrate the sample; V2= Volume of sodium thiosulphate in mL used to titrate the control; W: Weight of fat.

Acid index titration

The method used here is that of AFNOR [19]. The oil was dissolved in ethanolic-isobutanol (V/V) solution at a concentration of 40 g/L. 10 mL of alcoholic NaOH, 0.5 N were added into a 250 mL conical flask. 10 mL of the lipid solution, 40 g/L were added together with 2 drops of phenolphthalein this was titrated with a solution of HCL 1N of normality, NH+ while constantly shaking for about 30s until it became stably colourless. A control flask was prepared in the same way but the lipid solution was replaced by 10 mL of ethanolic-isobutanol solvent. Tubes were in triplicate. The following formula was used to calculate the acid index (AI):

$$\text{AI} = \frac{\text{NH}^+ \text{ X } (\text{V1} - \text{V2})}{\text{W}} \text{X } 4.00$$

AI: Acid index, NH^+ =1, V1= Volume of hydrochloric acid used to titrate the control; V2= Volume of hydrochloric acid used to titrate the sample; W: Weight of fat.

Induction of bone defects with oxidised palm oil and sucrose enriched diet

Two groups of normal rats were formed. The first group (SD, 12 animals) received the standard chow diet and water. The second group

(SD+POS, 40 animals) received the enriched diet supplemented with palm oil and water intake supplemented with 10% of sucrose. These animals were fed ad libitum for 112 days. Subsequently, the insulin sensitivity test (IST) and the oral glucose tolerance test (OGTT) were performed.

Insulin sensitivity test

To assess insulin sensitivity, after 12 hours of water-free fasting, the rats' blood glucose levels were measured (t = 0min) using a reagent strip and a glucometer (ACCU-CHEK®). Immediately thereafter, the animals received a dose of 0.15 IU/kg of insulin (branded Actrapid®) intraperitoneally. Blood glucose levels were determined at 10, 20, 30 and 60 minutes [20].

Oral glucose tolerance test

Oral glucose tolerance test was performed after an overnight fast (12 hours). Baseline blood glucose of each animal was measured (t = 0min). Rats were orally dosed with a D-glucose solution (5g/kg) and blood glucose concentrations were subsequently measured using glucometer at 30, 60, 120, and 180 min post-administration [21].

Fracture induction

The experimental equipment was first sterilized in 70% alcohol and the rats were anesthetized with Valium (10 mg/kg) and ketamine (30mg/kg) intraperitoneally. To expose the femur, the skin of the left thigh of each animal was incised with a scalpel blade, the muscle was opened and the femoral bone was exposed. A perforation (1.5 µm) was made in the femoral shaft using an electric drill (D-54343; FOHREN). After the fracture, sutures were applied and the wound was disinfected and treated with Betadine and penicillin (Baneocin) to prevent possible infection. This was repeated until the wound was completely healed [15].

Study design

Normal rats fed a standard chow were divided into two groups: a sham group (unfractured) and a fractured group (N-Fractured) both receiving distilled water (10 mL/kg). Fractured rats receiving the enriched diet supplemented with oxidised palm oil and water intake supplemented with sucrose (POS) were maintained on their diets and treated as follows: fractured group treated with distilled water (10 mL/kg) (FPOS+DW); Three fractured groups receiving *A. boonei* aqueous leaf extract at 50 mg/kg (FPOS+AB 50), 100 mg/kg (FPOS +AB 100) or 200 mg/kg (FPOS +AB 200). One group of rats receiving distilled water concomitantly with standard chow was added (FPOS1+DW).

The atherogenic index was determined using the following formula [23]:

$$Atherogenic\ Index = \frac{[\text{Total Cholesterol }]}{[\text{HDL} - \text{Cholesterol}]}$$

Evaluation of some markers of oxidative stress

The evaluation of the effects of the aqueous extract of the leaves of *A. boonei* on the oxidative parameters of insulin-resistant and fractured rats was obtained by measuring the levels of malondialdehyde (MDA) [24]; reduced glutathione (GSH) [25] as well as catalase activities [26] in the femoral homogenates.

Sample collection, serum, and homogenate preparation

On day 12, the animals were placed in metabolic cages and acclimatized for 24 hours. Food and water intakes and urinary excretion were assessed. The animals were fasted for 12-h then sacrificed under anesthesia with intraperitoneal injection of a combination of ketamin (30 mg/kg) and diazepam (10 mg/kg). The blood was collected in EDTA tubes for blood count analysis and in dry tubes for biochemical analysis. The latter was centrifuged at 3500 rpm for 15 min at 4°C and the serum obtained was stored at - 80°C for subsequent determination of alkaline phosphatase (ALP), calcium, phosphorus, glucose and Lipid profile (triglycerides; total cholesterol; HDL cholesterol and LDL-cholesterol).

After blood sampling, the animals were dissected, and the left and right femurs were removed. The fractured left femurs were X-rayed before being fixed in 4% formalin for histological sections of the fracture zone and the femoral heads. Right femoral heads were used for homogenates. 0.2 g of femur was homogenised in 3 mL phosphate buffered saline (PBS). The resulting homogenates were centrifuged at 3500 rpm for 25 minutes at 4°C. The supernatant was used to determine parameters including ALP, calcium, phosphorus, and some oxidative stress markers (malondialdehyde MDA, reduced glutathione GSH, and Catalase CAT).

Evaluation of hematological parameters

The blood count or hemogram was performed on rat whole blood collected in an EDTA tube using the Hematology counter type "Mindray-BC-5300" realised at the Laboratory of Hematology of the Central Hospital of Yaoundé. The parameters measured were as follows: White blood cells (WBC); red blood cells (RBC); Lymphocytes (LYM); monocytes (MON); Granulocytes (GRA); hemoglobin (HGB); Hematocrit (HCT); mean corpuscular volume (MGV) Platelets (PLA); Mean corpuscular hemoglobin concentration (MCHC); Red blood cell distribution index (RDI); Mean platelet volume (MPV); platelet distribution index (PDI).

Evaluation of biochemical parameters

To evaluate the effects of the aqueous extract of *A. boonei* on blood glucose levels, blood glucose was measured using the LABKIT kit (Canovelles-BARCELONA) following the protocol provided by the manufacturer. To evaluate the effects of the aqueous extract of *A. boonei* leaves on femoral fracture in rats, the serum and bone activities of alkaline phosphatase (ALP), calcium, phosphorus, triglycerides, total cholesterol and HDL cholesterol were determined using the LABKIT kit according to the manufacturer's instructions. LDL cholesterol was calculated using the formula written by Friedewald *et al*. [22]:

$$[LDL - Cholesterol] = [\text{Total Cholesterol}] - \frac{[\text{Triglyceride}]}{5} - [HDL - Cholesterol]$$

Radiological evaluation

To assess bone formation, the fractured left femur was X-rayed at the end of treatment using an X-ray machine. The radiological score was evaluated using a modified Lane and Sandhu scoring system [27].

Histopathological and histomorphometric analyses

The femurs were preserved in 10% formalin and demineralized in 10% hydrochloric acid and processed for routine histological examination. A microtome (Reichert-Jung 2030) was used to section the paraffin blocks containing the fractured bone portion to a thickness of 5 µm. Hematoxylin-eosin staining was performed. Microphotographs were taken using a camera with Digital Microscope Suite 2.0 imaging software.

Statistical analysis

The results were expressed as Mean ± Standard Error of the mean (SEM). Statistical analysis was performed using Graph Pad Prism 8.00 (GraphPad Software, San Diego, California, USA). Data were analyzed using one-way ANOVA followed by the Tukey's post hoc test. For the analysis of two distributions, the student's t test was used. Differences were considered significant at $p<0.05$.

RESULTS

Iodine, peroxide and acid index of thermo-oxidised palm oil

The iodine, peroxide and acid indexes of thermo-oxidised palm oil are shown in Table 1. Heating crude palm oil to 100°C showed a significant increase ($p<0.01$) in the peroxide and acid index. On the other hand, the iodine index decreased significantly ($p<0.05$).

Table 1: Quality parameters for thermo-oxidised palm oil

	Iodine index (g/100g of oil)	Peroxide index (meq/kg of oil)	Acid index (mgKOH/g oil)
RPO	33.78±6.59	2.367±1.01	5.16±0.51
POO1	16.05±3.11[a]	18.18±0.67[b]	12.01±1.16[c]
POO2	18.97±0.42[a]	20.18±3.50[b]	10.72±1.09[b]

Data are shown as mean ± SEM, n =3. [a]p<0.05, [b]p<0.01, [c]p<0.001: significant difference compared to RPO. RPO: Unheated red palm oil; POO1: heated and bleached red palm oil on the day of the test; POO2: represents the oil bleached one month ago.

Effects of aqueous extract of *Alstonia boonei* on body weight

Figure 1 illustrates the weight evolution of the animals during 112 days (16 weeks) of administration of oxidised palm oil-sucrose followed by the effect of treatment with aqueous extract of *A. boonei*. The figure shows that the diet enriched with oxidised palm oil-sucrose resulted in a gradual and significant increase in the body weight of the animals over the 112 days of the experiment. Fracture induction resulted in a decrease in body weight in all groups of animals compared with the normal control. Simultaneous administration of the supplement with the plant extract at doses of 50, 100 and 200 mg/kg induced a non-significant increase in the body weight of the animals compared with the fractured animals receiving the enriched diet (FPOS+DW).

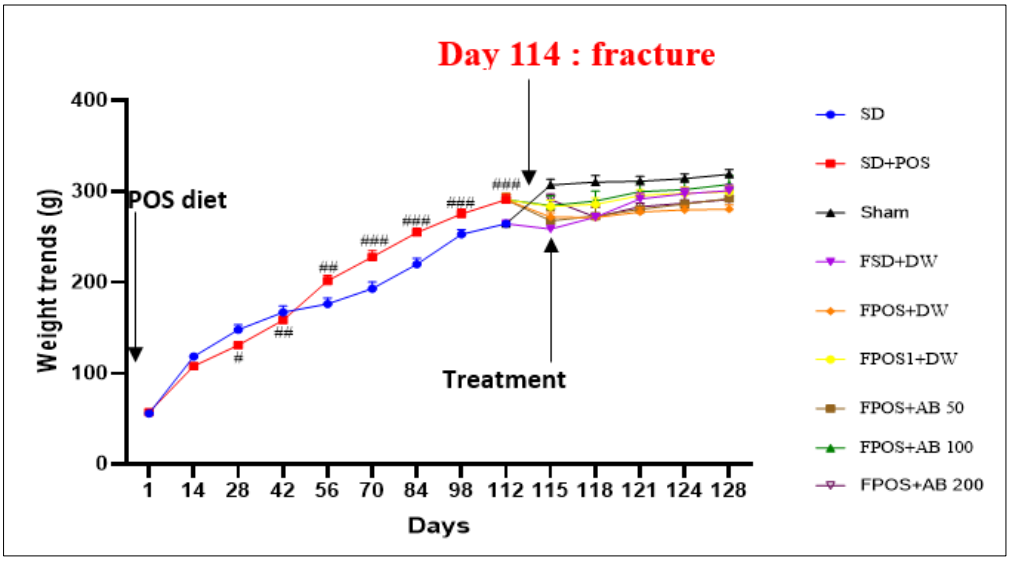

Figure 1: Effects of aqueous extract of *Alstonia boonei* on body weight in rats fed oxidised palm oil and sucrose

Data are shown as mean ± SEM, n =6. #p<0.05, ##p<0.01, ###p<0.001: significant difference compared to the initial point. SD : Animals fed the standard diet during 112 days; SD+POS : Animals fed oxidised palm oil in the standard diet and sucrose in the drinking water during 112 days; Sham: Animals not fractured (with simulated fractures) fed the standard diet and treated with distilled water (DW), FSD+DW; FPOS+DW: Fractured animals receiving enriched feed and treated with ED. FPOS1+ED: Fractured animals no longer receiving enriched feed and treated with distilled water; FPOS+AB 50, FPOS+AB 100, FPOS+AB 200: Fractured animals fed the enriched diet and treated with plant extract at doses of 50, 100 and 200 mg/kg respectively.

Effects of a diet enriched with thermo-oxidised palm oil and sucrose on insulin sensitivity and glucose tolerance tests

Figure 2 shows the impact of a diet enriched with oxidised palm oil and sucrose on insulin sensitivity (Figure 2A) and glucose tolerance (Figure 2B). The administration of insulin at a dose of 0.15 IU/kg to animals in the SD+POS group did not result in any significant difference in blood glucose levels at 60 min post-dosing compared with baseline. However, blood glucose levels in the SD+POS group remained 43.32% ($p<0.001$) higher than in the SD group at the end of the experiment. Oxidised palm oil and sucrose treated rats showed a significantly elevated ($p < 0.05$) blood glucose peak 60 min after oral glucose administration, with blood glucose levels still significantly higher at 2 h ($p < 0.01$) and 3 h ($p < 0.05$) compared to normal animals (Fig 2 B).

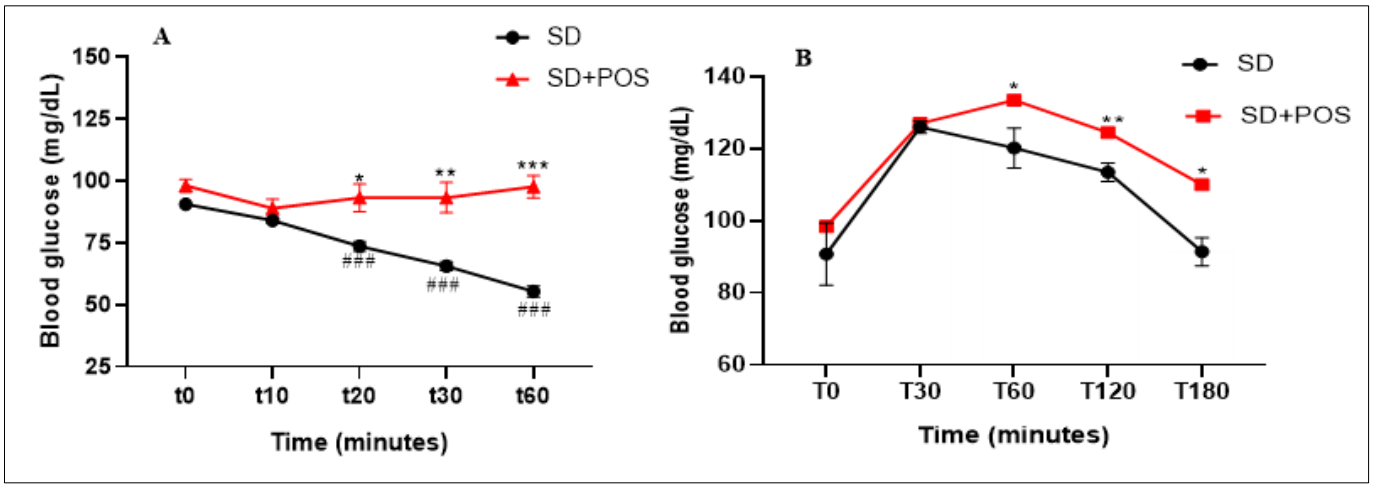

Figure 2: Effects of a diet enriched with oxidised palm oil and sucrose on insulin sensitivity (A) and glucose tolerance (B) tests.

Data are shown as mean ± SEM, n =6. *p<0.05, **p<0.01, ***p<0.001: significant differences between the SD+POS group and the SD group; ###p<0.001: significant difference compared to the initial point. SD: Animals fed the standard diet during 112 days; SD+POS: Animals fed oxidised palm oil in the standard diet and sucrose in the drinking water during 112 days.

Effect of the aqueous extract of *Alstonia boonei* on food and water intakes and urinary excretion

Table 2 represents the effect of *A. boonei* aqueous extract on food and water intakes, and urinary excretion. Pre-treatment supplementation with oxidised palm oil and sucrose resulted in a significant (p<0.001) decrease in food consumption and, a significant (p<0.001) increase in water intake and urinary excretion volume compared with the SD group. Simultaneous administration of the supplement and plant extract resulted in a significant decrease (p<0.001) in water intake and urinary excretion at all extract doses compared with the FPOS+ED group. Food consumption increased significantly at 100 mg/kg (p<0.001) and 200 mg/kg (p<0.01). Stopping the supplementation diet during the 14-day treatment period reversed these parameters.

Table 2: Effects of aqueous extract of *Alstonia boonei* on food and water intakes and urinary excretion volume

Quantity/24h/animal	Before treatment		After treatment						
	SD	SD+POS	Sham	FSD+DW	FPOS+DW	FPOS1+DW	FPOS+AB 50	FPOS+AB 100	FPOS+AB 200
Food (g)	19.14±0.58	7.77 ±0.73$^{\lambda}$	19.7±0.8	16.8+0.53	5.5±0.40***	15.63±0.55$^{###}$	6.92±0.69	10.83±0.55$^{\$\$\$}$	8.47±0.25$^{\$\$}$
Water (mL)	18.30 ±1.8	43.4±5.4$^{\lambda}$	29.3±1.9	29.28±0.41	68.65±0.51***	39.15±0.43$^{###}$	43.75±0.85$^{\$\$\$}$	28.50±2.66$^{\$\$\$}$	32±0.81$^{\$\$\$}$
Urine (mL)	9.69±0.59	18.83±2.19$^{\lambda}$	18.3±8.5	24.48±0.52	25.83±0.42	10.58±0.49$^{###}$	15.23±0.40$^{\$\$\$}$	12.58±0.93$^{\$\$\$}$	13.85±0.53$^{\$\$\$}$

Data are shown as mean ± SEM, n =6. $^{\lambda}$ p<0.001: significant difference between Animals fed the standard diet (SD) to the SD+POS. *** p<0.001: significant difference of Fractured animals receiving enriched feed (FPOS+ DW) to the fractured receiving standard diet (FSD+ DW); $^{###}$p<0.001: Significant difference of the FPOS1+ DW to the FPOS+ DW; $^{\$\$}$p<0.001, $^{\$\$\$}$p<0.001: Significant difference of the extracts at doses 50 (FPOS+AB 50), 100 (FPOS+AB 100) and 200 (FD+AB 100) mg/kg to the FPOOS+ED. SD : Animals fed the standard diet during 112 days; SD+POS : Animals fed oxidised palm oil in the standard diet and sucrose in the drinking water during 112 days; Sham: Animals not fractured (with simulated fractures) fed the standard diet and treated with distilled water (DW), FSD+ DW: fractured receiving standard diet and treated with distilled water ; FPOS+ DW: Fractured animals receiving enriched feed and treated with DW. FPOS1+ DW: Fractured animals no longer receiving enriched feed and treated with distilled water; FPOS+AB 50, FPOS+AB 100, FPOS+AB 200: Fractured animals fed the enriched diet and treated with plant extract at doses of 50, 100 and 200 mg/kg respectively.

Effects of aqueous extract of *Alstonia boonei* on glycemia and lipid profile of fractured rats fed oxidised palm oil and sucrose

Table 3 shows the effects of *A. boonei* aqueous extract on the blood glucose and lipid profiles of animals supplemented with oxidised palm oil-sucrose. The ingestion of a diet supplemented with oxidised palm oil-sucrose resulted in a significant increase in fasting glucose by 55%.00 (p<0.001) compared with fracture animal receiving the standard diet. The administration of the extract at the doses of 50, 100 and 200 mg/kg concomitantly with the supplement significantly (p<0.001) decreased blood glucose level respectively by 25.60 %, 21.53% and 16.01% compared to the POS-fractured group.

Fractured animals fed the oxidised palm oil-sucrose supplemented diet showed a significant increase in total cholesterol by 41.92 % (p<0.001); triglycerides by 278.83 % (p<0.001), LDL-Cholesterol by 31.76 % (p<0.05) and atherogenic index by 201.15 % (p<0.01) compared to fractured animals fed the standard diet. However, there was a significant decrease in HDL of 57.22% (p<0.001) in these animals. Co-administration of the plant extract with the oxidised palm oil-sucrose supplement resulted in a significant reduction in total cholesterol, atherogenic index and a significant increase in HDL-Cholesterol compared to the fractured POS-group.

Table 3: Effects of *Alstonia boonei* aqueous extract on blood glucose and lipid profiles

	FSD+DW	FPOS+DW	FPOS1+DW	FPOS+AB 50	FPOS+AB 100	FPOS+AB 200
Blood glucose level (mg/dL)	74.00±0.36	114.7±0.76***	100.3±2.01$^{###}$	85.33±1.64$^{\$\$\$}$	90.00±1.15$^{\$\$\$}$	96.33±2.69$^{\$\$\$}$
Total Cholesterol (mg/dL)	191.30±4.87	271.50±3.52***	215.20±7.54$^{##}$	224.80±6.02$^{\$}$	175.00±1.46$^{\$\$\$}$	174.70±20.39$^{\$\$\$}$
Triglycerides (mg/dL)	32.89±0.91	124.60±6.36***	71.74±9.59$^{###}$	79.96±2.51$^{\$\$\$}$	59.35±1.18$^{\$\$\$}$	68.11±0.54$^{\$\$\$}$
HDL-Cholesterol (mg/dL)	32.89±1.79	14.07±0.71***	14.83±1.21	25.41±1.99$^{\$\$}$	24.72±0.45$^{\$\$}$	21.56±2.15$^{\$}$
LDL-Cholesterol (mg/dL)	176.60±3.47	232.70±5.53*	175.00±9.33	164.40±4.96$^{\$}$	134.00±3.22$^{\$\$\$}$	141.90±24.87$^{\$\$\$}$
Atherogenic index	6.07±0.41	18.28±0.84***	16.22±0.75	7.44±0.92$^{\$\$\$}$	8.377±0.30$^{\$\$\$}$	10.76±2.15$^{\$\$\$}$

Data are shown as mean ± SEM, n=6, *p<0.05, ***p<0.001: Significant difference of the FPOS+DW to the FSD+DW; $^{##}$p<0.01, $^{###}$p<0.001: Significant difference of the FPOS1+DW to the FPOS+DW; $^{\$}$p<0.05, $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001: Significant difference of the extracts at doses 50 (FPOS+AB 50), 100 (FPOS+AB 100) and 200 (FPOS+AB 200) mg/kg to the FPOS+DW. FSD+DW: fractured receiving standard diet and treated with distilled water; FPOS+DW: Fractured animals receiving enriched feed and treated with DW. FPOS1+DW: Fractured animals no longer receiving enriched feed and treated with distilled water; FPOS+AB 50, FPOS+AB 100, FPOS+AB 200: Fractured animals fed the enriched diet and treated with plant extract at doses of 50, 100 and 200 mg/kg respectively.

Effects of *Alstonia boonei* treatment on hematological parameters of fractured rats fed oxidised palm oil and sucrose

The effects of the aqueous extract of *Alstonia boonei* on hematological parameters are summarised in Table 4. Fracture induced in animals receiving the oxidised palm oil-sucrose supplement did not cause significant changes in haematological parameters compared with the FSD+DW group. Administration of *Alstonia boonei* plant extract to animals receiving the oxidised palm and sucrose supplement for 14 days at a dose of 50 mg/kg induced a significant decrease (p<0.05) in red blood cell count and a significant increase (p<0.01) in white blood cell count compared with animals in the FPOS+DW group. On the other hand, administration at a dose of 200 mg/kg induced a significant decrease in hematocrit and platelet levels compared to the FPOS+DW group.

Table 4: Hematological parameters after treatment with plant extract

Paramètres	Sham	FSD+DW	FPOS+DW	FPOS1+DW	FPOS+AB 50	FPOS+AB 100	FPOS+AB 200
RBC (10^6 / mm^3)	11.41±0.25	8.17±0.00	8.64±0.02	8.40±0.80	6.92±0.00$^{\$}$	9.60±0.17	8.00±0.05
WBC (10^3 /mm^3)	3.64±0.23	9.23±0.002	8.49±0.005	10.80±2.07	14.40±0.11$^{\$\$}$	13.00±1.38	12.70±0.23
HGB (g/dL)	18.38 ±0.37	13.66±0.03	14.12±0.06	12.30±0.51$^{\#}$	10.65±0.02$^{\$\$\$}$	16.30±0.57$^{\$\$}$	9.90±0.17$^{\$\$\$}$
HCT (%)	60.08±1.60	42.92±0.01	49.62±0.00**	43.60±2.30$^{\#\#}$	32.65±0.20$^{\$\$\$}$	48.15±0.72	33.15±0.02$^{\$\$\$}$
PLA (10^3 /mm^3)	354.00±18.75	891.5±0.86	846.5±0.86	329.0±51.9$^{###}$	306.0±0.57$^{\$\$\$}$	403.0±9.81$^{\$\$\$}$	271.5±28.00$^{\$\$\$}$

Data are shown as mean ± SEM, n =6, **p<0.01: Significant difference of the FPOS+DW to the FSD+DW; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{###}$p<0.001: Significant difference of the FPOS1+DW to the FPOS+DW; $^{\$}$p<0.05, $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001: Significant difference of the extracts at doses 50 (FPOS+AB 50), 100 (FPOS+AB 100) and 200 (FPOS +AB 200) mg/kg to the FPOS+DW. Sham: Animals not fractured (with simulated fractures) fed the standard diet and treated with distilled water (DW), FSD+DW: fractured receiving standard diet and treated with distilled water; FPOS+DW: Fractured animals receiving enriched feed and treated with DW. FPOS1+DW: Fractured animals no longer receiving enriched feed and treated with distilled water; FPOS+AB 50, FPOS+AB 100, FPOS+AB 200: Fractured animals fed the enriched diet and treated with plant extract at doses of 50, 100 and 200 mg/kg respectively. WBC: White blood cells; RBC: Red blood cells; HGB: Hemoglobin; HCT: Hematocrit; PLA: Platelets.

Effects of *Alstonia boonei* treatment on some biochemical parameters of bone function of fractured rats fed oxidised palm oil and sucrose

Figure 3 illustrates the effects of the aqueous extract of *A. boonei* leaves on bone (Figure 3 A) and serum (Figure 3B) alkaline phosphatase activity. A significant increase of 43.75 % (p<0.05) increase in bone ALP in fractured rats fed oxidised palm oil and sucrose compared with fractured animals fed the standard diet. The fractured rats receiving standard chow for fourteen days treatment showed a significant reduction in ALP compared with fractured receiving continuously oxidised palm oil and sucrose. Administration of the plant extract at a dose of 100 mg/kg caused a significant decrease in ALP of 40.23% compared with the fractured group receiving the oxidised-sucrose. With regard to serum ALP, a significant increase in alkaline phosphatase (p<0.001) was observed in fractured animals receiving the standard diet supplemented with palm oil compared with fractured animals receiving the standard diet. Concomitant administration of the plant extract with the oxidised palm oil-sucrose supplement induced a significant decrease in ALP in animals treated at doses of 50 mg/kg and 100 mg/kg compared with animals in the FPOS+DW group. However, at a dose of 200 mg/kg, a significant increase in serum ALP was observed.

Compared with the sham group fractured rats fed oxidised palm oil and sucrose showed a decrease (p>0.05) in bone and serum calcium. Whatever the dose the aqueous extract of *A. boonei* leaf significantly (p<0.001) increased bone calcium; whereas the extract caused a significant decrease in serum calcium compared to standard chow group.

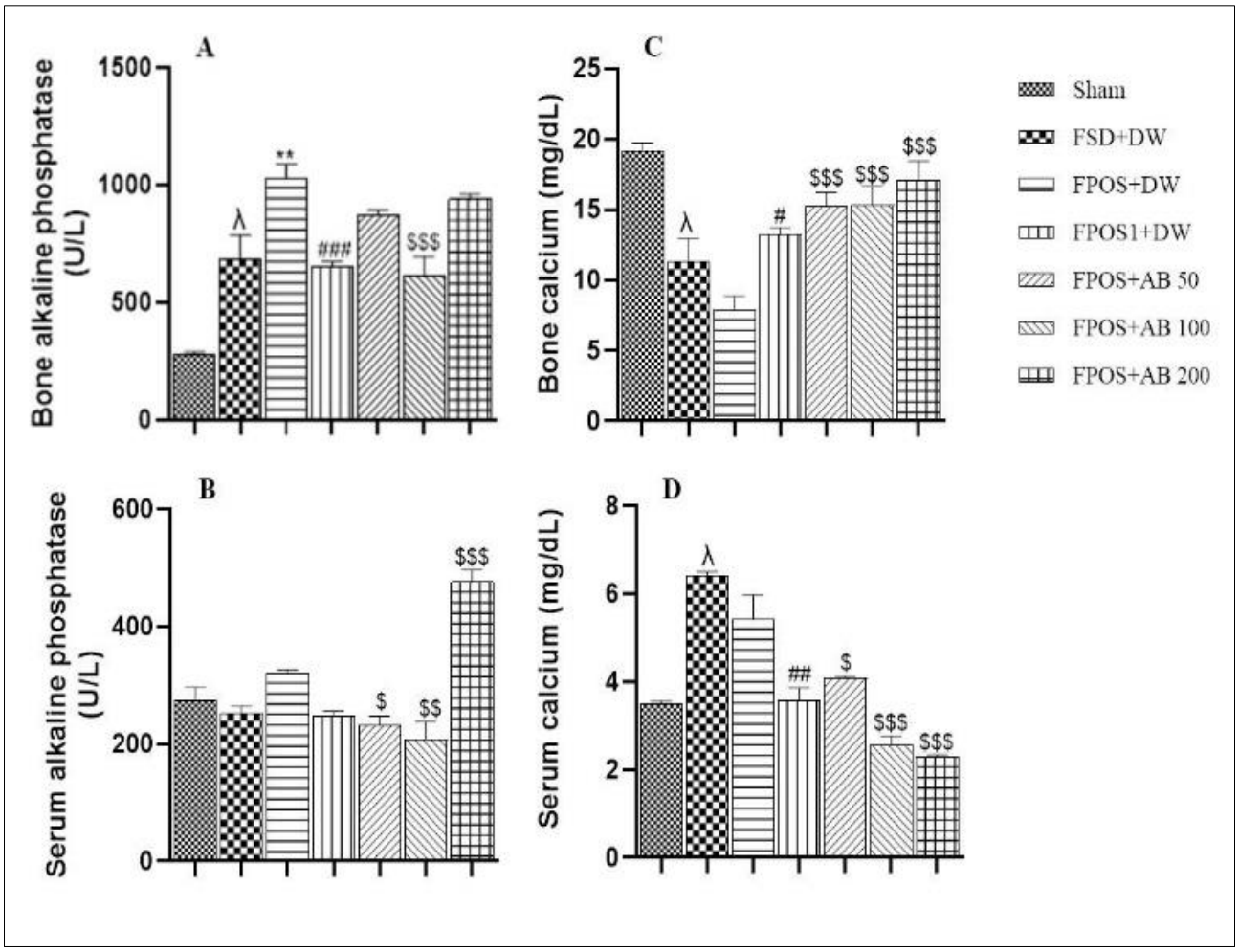

Figure 3: Effects of *Alstonia boonei* treatment on fractured rats fed a diet enriched with oxidised palm oil and sucrose.

Data are shown as mean ± SEM, n =6, $^{\lambda}p<0.05$: Significant difference of the FSD+DW to the Sham; $^{**}p<0.01$: Significant difference of the FPOS+DW to the FSD+DW; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$: Significant difference of the FPOS1+DW to the FPOS+DW; $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$: Significant difference of the extracts at doses 50 (FPOS+AB 50), 100 (FPOS+AB 100) and 200 (FPOS +AB 200) mg/kg to the FPOS+DW. Sham: Animals not fractured (with simulated fractures) fed the standard diet and treated with distilled water (DW), FSD+DW: fractured receiving standard diet and treated with distilled water; FPOS+DW: Fractured animals receiving enriched feed and treated with DW. FPOS1+DW: Fractured animals no longer receiving enriched feed and treated with distilled water; FPOS+AB 50, FPOS+AB 100, FPOS+AB 200: Fractured animals fed the enriched diet and treated with plant extract at doses of 50, 100 and 200 mg/kg respectively.

Effects of *Alstonia boonei* treatment on some oxidative stress parameters of fractured rats fed oxidised palm oil and sucrose

The effects of the aqueous extract of *A. boonei* leaves on some parameters of oxidative stress in the femur are presented in Figure 4. Ingestion of oxidised palm oil-enriched diet and sucrose resulted in a significant increase of malondialdehyde levels by 29.27 % ($p<0.001$) in the femur compared to fractured animals receiving the standard diet. Once-daily administration of the extract for two weeks significantly reduced ($p<0.001$) MDA levels by 44.86; 32.43%; 50.91% respectively at the doses of 50 mg/kg, 100 mg/kg and 200 mg/kg. Fractured rats fed oxidised palm oil and sucrose also showed a decrease ($p>0.05$) in reduced glutathione (GSH) compared with animals in the fractured group receiving the standard diet. There was also a significant increase ($p<0.05$) in GSH in fractured animals in which the oil-enriched diet was discontinued during the treatment period compared with animals in the (FPOS+DW) group. Administration of the extract at a dose of 100mg/kg significantly increased GSH by 25,39 % ($p<0.01$). Fractured animals receiving the enriched diet showed a significant increase in catalase compared with the standard chow group (Figure 4 C). Administration of the extract at all doses significantly ($p<0.01$) reduced femoral catalase activity compared with the fractured rats fed oxidised palm oil and sucrose groups.

Effects of aqueous extract of *Alstonia boonei* on histological sections of rats fed a diet enriched with palm oil and sucrose

Figure 5 shows radiographs of the femurs of rats. A completely fractured bone with the appearance of a bone callus over the fracture site was observed in rats fed the enriched diet, compared with fractured rats fed the standard diet; which have a partially fractured bone with the appearance of a very fibrous bone callus covering the fracture site. The radiographs of the rats treated with the plant extract at doses of 50, 100 and 200 mg/kg show a denser and less fibrous bone callus than that of the insulin-resistant fracture group.

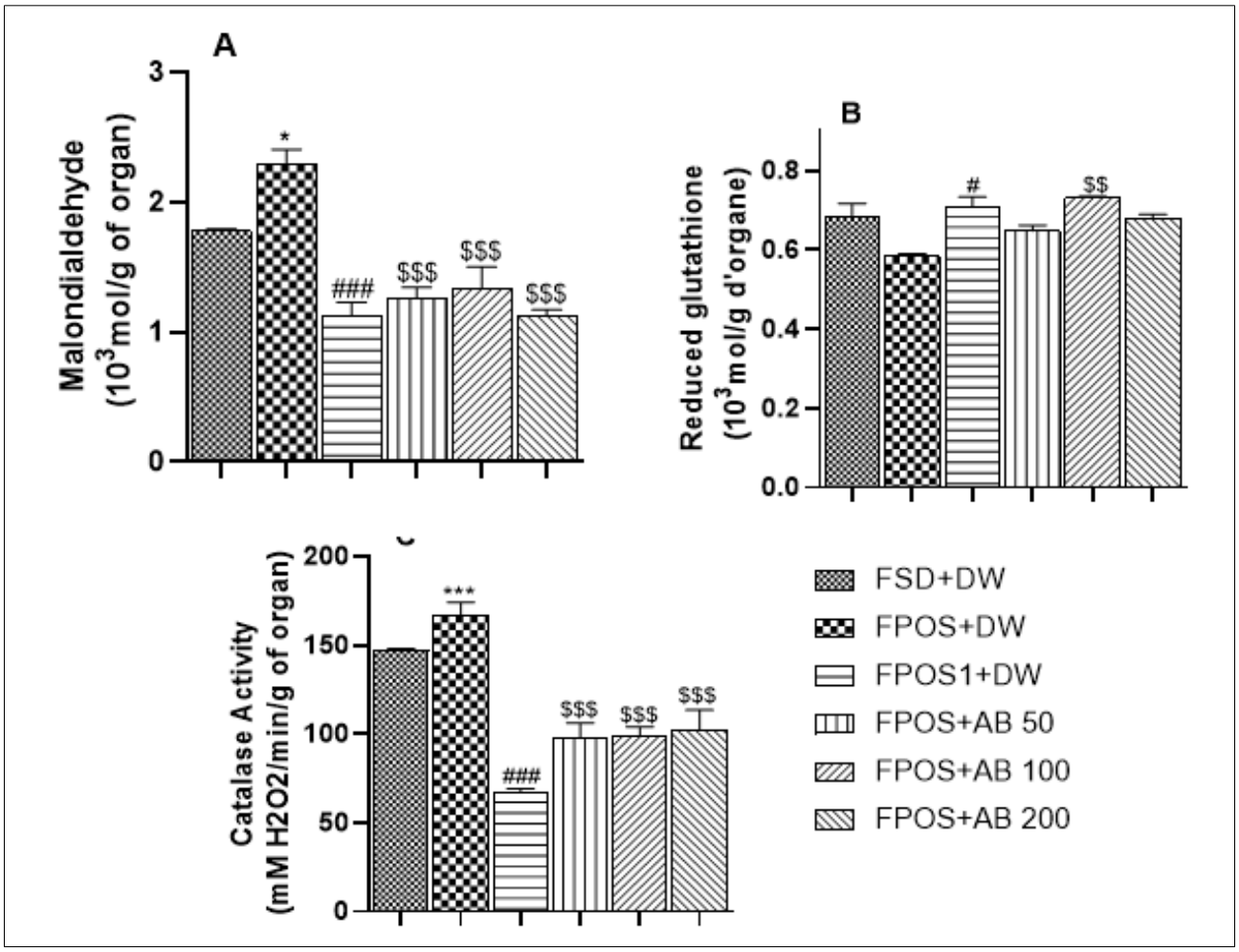

Figure 4: Effects of *Alstonia boonei* treatment on MDA (A) GSH (B) and catalase activity (C) of fractured rats fed a diet supplemented with oxidised palm oil and sucrose.

Data are shown as mean ± SEM, n =6, $^{*}p<0.05$, , $^{***}p<0.001$: Significant difference of the FPOS+DW to the FSD+DW; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$: Significant difference of the FPOS1+DW to the FPOS+DW; $^{\$\$}p<0.01$,$^{\$\$\$}p<0.001$: Significant difference of the extracts at doses 50 (FPOS+AB 50), 100 (FPOS+AB 100) and 200 (FPOS +AB 200) mg/kg to the FPOS+DW, FSD+DW: fractured receiving standard diet and treated with distilled water; FPOS+DW: Fractured animals receiving enriched feed and treated with DW. FPOS1+DW: Fractured animals no longer receiving enriched feed and treated with distilled water; FPOS+AB 50, FPOS+AB 100, FPOS+AB 200: Fractured animals fed the enriched diet and treated with plant extract at doses of 50, 100 and 200 mg/kg respectively.

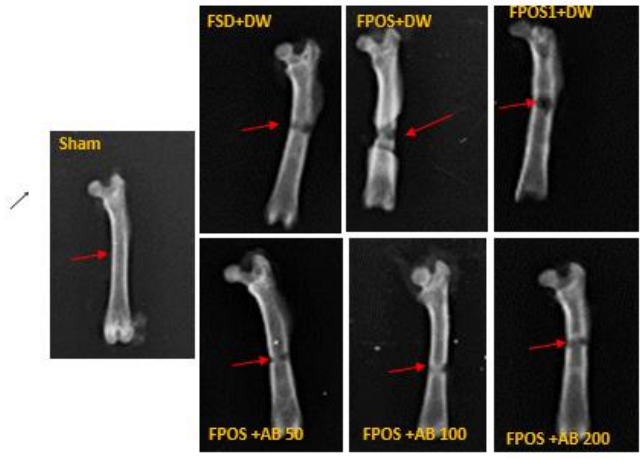

Figure 5: Effects of aqueous extract of *Alstonia boonei* on bone X-ray images

FSD+DW: fractured receiving standard diet and treated with distilled water; FPOS+DW: Fractured animals receiving enriched feed and treated with DW. FPOS1+DW: Fractured animals no longer receiving enriched feed and treated with distilled water; FPOS+AB 50, FPOS+AB 100, FPOS+AB 200: Fractured animals fed the enriched diet and treated with plant extract at doses of 50, 100 and 200 mg/kg respectively.

Effects of aqueous extract of *Alstonia boonei* on the microarchitecture of the fractured femur of rats fed a diet enriched with palm oil and sucrose

Figure 6 shows the effects of the aqueous extract of the whole plant of *A. boonei* on the microarchitecture of bone tissue following femur fractures in animals supplemented with oxidised palm oil-sucrose. In fractured rats receiving the standard diet the femur showed an ossified callus, with a disorganised and compact bone arrangement. In the fractured animals receiving the diet enriched with oxidised palm oil-sucrose, the femur showed an ossified callus with a disorganised bone arrangement and a less dense and sparse callus structure at the fracture site compared with fractured receiving the standard died. The plant extract at all doses corrected these abnormalities and showed a well organised and fairly compact structure. However, it should be noted that the cortical bone of animals receiving the extract at a dose of 100 mg/kg was close to that of non-fractured animals.

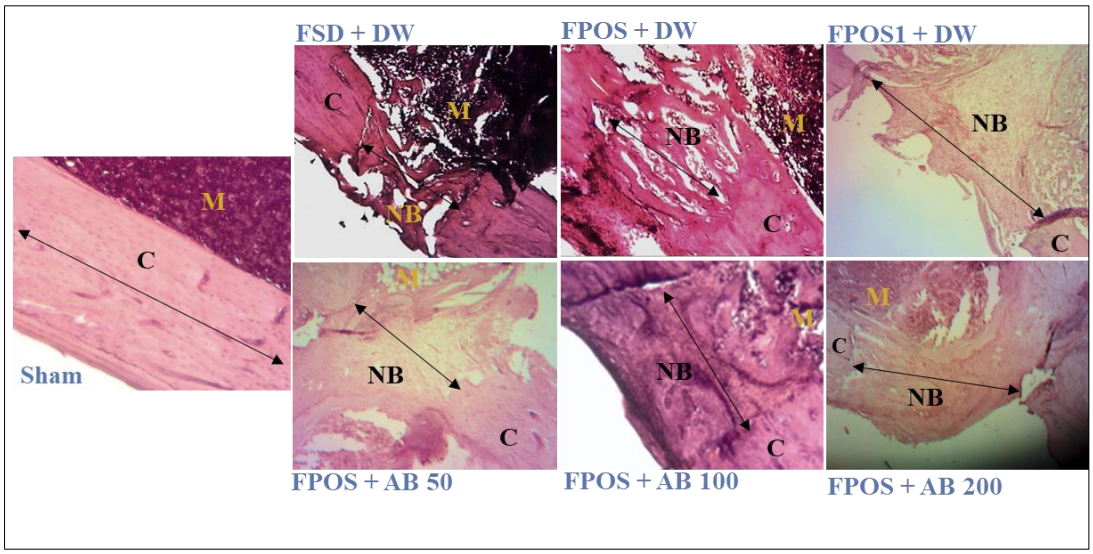

Figure 6: Effects of aqueous extract of *Alstonia boonei* on bone tissue microarchitecture (HE X 40)

FSD+DW: fractured receiving standard diet and treated with distilled water; FPOS+DW: Fractured animals receiving enriched diet and treated with DW. FPOS1+DW: Fractured animals no longer receiving enriched feed and treated with distilled water; FPOS+AB 50, FPOS+AB 100, FPOS+AB 200: Fractured animals fed the enriched diet and treated with plant extract at doses of 50, 100 and 200 mg/kg respectively. C: Cortical; M: Morrow; NB: New bone.

DISCUSSION

Palm oil is the most produced and consumed oil in the world [28]. In Africa, this oil in our households is often bleached and reheated several times during use. Red palm oil is made up of 50% saturated fatty acid and 50% unsaturated fatty acid [29]. The saturated bonds can withstand high temperatures. However, the double bonds are more likely to open and combine with oxygen. Our palm oil, which has been bleached and heated several times, has been classified as a thermo-oxidised oil after a series of chemical quality tests, such as the iodine, peroxide and acid index. The iodine value was significantly lower in the heated oil than in the unheated red oil. The iodine value is used to determine the amount of unsaturated fatty acids in the oil. The higher the iodine number, the more unsaturated the oil. When palm oil was heated to high temperatures, the double bonds of the unsaturated fatty acids were able to break, resulting in a reduction in the number of unsaturated fatty acids and therefore a reduction in the amount of iodine fixed, which explains the lower iodine value of the heated oil compared to the unheated oil. The peroxide value is an indication of the amount of hydroperoxides and peroxides present in the oil. These compounds are formed by oxidation of lipids. It is used to determine the onset of oxidation of an oil because the oxidation process of an oil takes place in three stages: initiation, propagation and termination [30]. The high peroxide value of our heated oil would be due to the heating temperature of our oil. Heat promotes the oxidation of fatty acids, which increases the formation of peroxides [31]. The value of the heated palm oil was higher than 15 meq of O2/kg of oil, which is the maximum content for cold-pressed and virgin oils [32]. This result indicates that this heated palm oil has been denatured during heating and is therefore unfit for consumption. Oils generally contain small amount of free fatty acids, and these fatty acids are responsible for the acidity and oxidizability of oils, produce unpleasant odours when exposed to air. The acid number measures the amount of these free fatty acids in the oil [32]. The heated palm oil had a higher acid value than unheated palm oil. This should be explained by the fact that heating the oil increases the free fatty acids in the oil, which can easily oxidise when expose to air [32]. The iodine value of the heated palm oil was greater than 4.0 mg KOH/g of oil, which is the maximum level for cold-pressed and virgin oils. This showed that the oil was suitable for human consumption from the outset. Administration of oxidised palm oil and sucrose in drinking water induced a significant increase in body mass associated with onset of impaired glucose tolerance and reduced insulin sensitivity. These results are in line with those of Ngueguim *et al* [16] who demonstrated that this diet led to a metabolic disorder. The insulin resistance induced by the supplement is mainly due to the presence of fructose contained in sucrose, which is more harmful than glucose in terms of lipogenesis and insulin resistance [33], and to the presence of fatty acids, mainly palmitic acid in oxidised palm oil [34]. Indeed, the mechanism by which fructose establishes insulin resistance when consumed chronically is thought to involve the metabolism of fructose into triglycerides in the liver [35]. Dyslipidemia and an increase in the atherogenic index were also observed in this study. These results once again confirm the lipogenic and lipotoxic nature of fructose, which is responsible for the insulin resistance induced by this food. It is therefore possible that oxidised palm oil and sucrose act synergistically to increase the lipogenic nature of the supplement and thus increase insulin resistance. Prior to fracture induction, there was a reduction in food intake associated with polydipsia and polyuria throughout the treatment period. Assuming that the oxidised palm oil supplement has a bad taste and unpleasant odour, and therefore an anorectic effect, then the animals would have lost their appetite due to the unpleasant odour of the oxidised palm oil, leading them to preferentially prefer sugar water, thus compensating the anorectic effect. Administration of the aqueous extract of the *A. boonei* plant induced an increase in food consumption and a decrease in water intake, with a consequent reduction in diuresis. These results suggest that the plant extract would have orexigenic effect, which explains the animals' return to feed and the consequent reduction in water intake and diuresis. Insulin resistance is an important risk factor for fracture [36]. Insulin resistance significantly weakens bones considerably to the point where a minor shock can lead to a fracture.

Fracture disrupts or alters the target responsible for the production of haematopoietic cells. This explains the increase in white blood cell (WBC) and platelet (PLA) levels observed in fractured animals fed the standard diet compared with non-fractured animals. These results are similar to those obtained by Ngueguim *et al*. [15]. Bone has a natural repair capacity. The increase in WBC is indicative of an inflammatory process which is observed during natural bone consolidation process [37]. In the event of a fracture, the blood vessels supplying the affected bone are damaged and bleeding occurs. Platelets increase in the bloodstream and at the fracture site and stop the bleeding by coagulating the blood and forming a haematoma in the bone, which is the first stage of bone repair [38]. Administration of the plant extract reduced blood platelet levels, indicating an advanced stage of bone repair.

Alkaline phosphatase (ALP) is a marker of bone formation [39]. It is secreted by osteoblasts and plays a role in bone mineralisation [40]. The increase in bone alkaline phosphatase in fractured animals receiving the standard diet indicates natural bone repair. The significant increase in ALP in fractured rats receiving the oxidised palm oil-sucrose enriched diet compared with fractured rats receiving the standard diet suggests a natural but slow bone mineralisation repair, since as according to Sousa *et al* [41] ALP levels decrease after fracture healing. Hyperlipidemia increases the production of oxidised lipids, which in bone induce osteoclastogenesis and attenuate the effects of BMP-2, a protein whose expression induces osteogenesis [42]. Aqueous extract of *A. boonei* at a dose of 100 mg/kg resulted in a significant decrease in ALP. This reduction suggests that the extract induces bone healing. In addition, some studies have confirmed a drop in alkaline phosphatase levels after fracture healing [41].

Calcium and phosphorus are necessary for bone formation and are stored in the bone in the form of hydroxyapatite, which gives bone its rigidity. Administration of the extract at any dose resulted in a significant increase in calcium. These results are attributed to the presence in the aqueous extract of *A. boonei* of calcium and phosphorus (which will form hydroxyapatite), magnesium and flavonoids that increase the functions of collagen synthesis and mineralisation [43]. These results are once again confirmed by the bone repair observed after histological and radiological analysis with a marked effect at the 100 mg/kg dose. At this dose of extract, a very dense cortical bone was observed, close to that of unfractured animals. It has been reported that reactive oxygen species (ROS) are a risk factor for fracture [44] and, that ROS inhibit osteoblasts differentiation and promote apoptosis [45]. In this study, the presence of ROS was confirmed by the increase in malondialdehyde and catalase activity. The origin of this stress would be due to the damage caused by the fracture (destruction of bone tissue) associated with an increase in the expression of NADPH oxidase, an enzyme responsible for the formation of free radicals in the mitochondrial chain [46]. This oxidative stress could be due to an exogenous input of oxidised palm oil which, when heated to high temperatures, loses its antioxidants (carotenoids and vitamin E), when heated to high temperatures, exposing the palm oil to reactions with atmospheric oxygen. ROS are also thought to activate osteoclastogenesis and increase the RANK-L/RANK ratio [6], while also inhibiting rapid bone repair. These results corroborate those of the histology of the fracture site of animals receiving the oxidised palm oil-sucrose supplement, which showed disorganised bone formation with sparse architecture compared with fractured animals receiving the standard diet. Administration of the aqueous extract of *A. boonei* significantly reduced MDA levels, improved catalase activity and significantly increased reduced glutathione at a dose of 100 mg/kg, suggesting the antioxidant properties of this extract. This biological activity is attributed to the presence of quercetin, a flavonoid present in the extract, which inhibits the production of ROS. This improvement is justified by the marked bioactive effects of the aqueous extract of *A. boonei* on the reconstitution of bone microarchitecture.

CONCLUSION

Supplementation with thermos-oxidised palm oil and sucrose induced a metabolic disturbance manifested by reduced insulin sensitivity, glucose intolerance with dyslipidaemia, reduced food intake, polydipsia and polyuria. Changes in calcium, phosphorus and alkaline phosphatase activity and oxidative stress were observed after fracture. In addition, rats with fractures fed a diet enriched with thermos-oxidised palm oil and sucrose showed a delay in the natural healing of the fractured bone compared with those fed the standard diet. Treatment with the aqueous extract of *A. boonei* leaf, given concomitantly with the diet, improved lipid profile, food and water consumption and reduced diuresis. Histological sections and radiological images also showed bone consolidation. These effects are thought to result from metabolites such as flavonoids in the extract with osteoinductive activity, and calcium and phosphorus (responsible for mineralisation) in the plant extract. The effects observed were marked at a dose of 100 mg/kg. These results suggest that the aqueous extract of *A. boonei* has osteoinductive effects in animals fed a diet enriched with thermos-oxidised palm oil and sucrose in the drinking water.

Conflict of interest

The authors declare that they have no conflict of interest.

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None declared.

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