Pterocarpus soyauxii **(Fabaceae) heartwood aqueous extract exhibits anti-osteoporotic activities in a postmenopausal-like model**

Abstract

Previous studies showed that *Pterocarpus soyauxii* (*P. soyauxii*) exhibits estrogenic activities ease menopausal disorders. The objective of this study was to evaluate anti-osteoporotic activities of the aqueous extract of *P. soyauxii* heartwood in ovariectomized (Ovx) Wistar rats. To achieve this, an 84-day postmenopausal osteoporosis model was used. Twenty-five female rats were ovariectomized and 5 others were sham-operated (Sham). After 84 days of hypoestrogenism, Ovx animals were divided into 5 groups including a group receiving distilled water at 10 mL/kg, a group receiving estradiol valerate (E_2V) at 1 mg/kg, and three groups receiving *P. soyauxii* extract at 100, 200, and 300 mg/kg; Sham-operated animals received vehicle (10 mL/kg). After 28 days of treatment, animals were sacrificed. Blood was collected in EDTA tubes for blood count and in dry test tubes. Some femoral and seric biochemical analyses were carried out. The relative mass of both tibia and femur, and femoral density were assessed. As result, oophorectomy significantly increased the level of white blood cells (WBC) ($p < 0.01$), MDA ($p < 0.01$), nitrites ($p < 0.001$), and urinary calcium/urinary creatinine ratio ($p < 0.01$). Ovx animals presented a low femoral calcium and phosphorus levels ($p < 0.05$) likewise ALP activity in both serum and femur compared to sham group. Thus, they also presented numerous resorption lacunae in the tibia and femur and a disorganization of tibia trabecular bone. *P. soyauxii* extract at 300 mg/kg significantly decreased WBC ($p < 0.05$), MDA ($p < 0.01$), and nitrites ($p < 0.001$) compared to vehicle. At the dose of 200 mg/kg, *P. soyauxii* extract significantly increased femoral calcium ($p < 0.05$), seric phosphorus ($p < 0.01$), and ALP activity ($p < 0, 05$) in both femur and serum, as well as relative femoral mass ($p < 0.05$) and density ($p < 0.001$). Furthermore, the plant extract at 200 and 300 mg/kg reduced resorptive lacunae and reconstituted trabecular bone in Ovx animals. Overall, aqueous extract of *P. soyauxii* exhibits anti-osteoporotic activities in a postmenopausal-like model in Wistar rats.

Key words: *P. soyauxii*, anti-osteoporotic, postmenopausal, rat.

1- Introduction

Osteoporosis is a systemic disorder characterized by a reduction in bone mass and microarchitectural deterioration of bone tissue, resulting in skeletal fragility [1]. Known as a multifactorial disease, its susceptibility is determined by genetic influences, environmental factors, and sex hormone status among others [2]. The ever-increasing aging of the world's population makes osteoporosis a major public health problem [3]. Estrogen deficiency in postmenopausal women, however, is known to be an important factor in the pathogenesis of osteoporosis [4]. Prevention and management of this condition are based primarily on

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Hormone Replacement Therapy (HRT) [5]. Despite the positive effects associated with HRT for osteoporosis, the Women's Health Initiative (WHI) trial reported a risk of cardiovascular diseases and breast cancer associated with this healing [6]. Around the world and particularly in developing countries, herbs are used as a therapeutic alternative to conventional medicine [7]. Plants commonly used to prevent menopausal disorders have among other estrogenic activities that are linked to their richness in a class of molecules called phytoestrogens. These molecules have been shown to possess anti-osteoporotic effects without the adverse effects associated with estrogens, such as estrogen-dependent cancers, reported in experimental studies [8-9]. Previous studies showed that *P. soyauxii*, the subject of this study exhibits estrogen-like effects in a postmenopausal model [10]. Indeed, this study confirmed the ethnobotanical uses of this plant to manage uro-genital issues in women [11-15]. Actually, the aqueous extract of the plant contains phytoestrogens like pterostilben and linoleic acid, thus it reduced menopausal impairment like vaginal atrophy and metabolic syndrome. Nevertheless, effects on postmenopausal osteoporosis have not yet been evaluated. Thus, this study aimed to evaluate the anti-osteoporotic activities of *P. soyauxii* heartwood aqueous extract in a postmenopausal-like model induced by ovariectomy in rat.

2- Material and methods

2.1- P. soyauxii extraction and Ethics

The aqueous extract of *P. soyauxii* was prepared according to the protocol described by Mengue *et al*. [10]. All experiments were conducted following the principles and procedures of the European Union on Animal Care (CEE Council 86/609) guidelines adopted by the Cameroon Institutional National Ethics Committee, Ministry of Scientific Research and Technology Innovation (Reg. number FWA-IRD 0001954).

2.2- Animal material

Wistar strain female rats aged 10-12 weeks and weighing between 130-150 g were used. They were housed in plastic cages of 5 animals per cage with free access to tap water and soy-free chow. The animals were ovariectomized using a dorsal approach [9,15].

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2.3- Experimental design

Thirty rats were used. Twenty-five rats were ovariectomized and the others were the sham-operated (Sham). 84 days after oophorectomy, the animals were divided into 6 groups of 5 animals each and were treated daily for 28 days as follows: the sham group received distilled water (10 mL/kg) and an Ovx animals received respectively distilled water (10 mL/kg), E_2V (1 mg/kg), and *P. soyauxii* at 100, 200 and 300 mg/kg. Before sacrifice, fasted animals were individually housed in a metabolic cage for 24 h. A urine sample was collected and acidified with 2 mL of 1 mol/L HCl. Collected urine samples were used for the determination of creatinine, calcium, inorganic phosphorus, magnesium. Thus, some osteolysis indexes (Urinary-calcium/Urinary-creatinine; Urinary-magnesium/Urinary-creatinine) were calculated. Vaginal smears were also carried out. Arteriovenous blood was collected both in EDTA tubes for blood count and in dry tests tubes for centrifugation (3500pm for 15 min). Calcium, magnesium, creatinine, inorganic phosphorus (IP) levels, and alkaline phosphatase activity (ALP) were assessed in serum. Femur and Tibia were collected and weighed. 0.2 g of the head of femur was homogenized in 2 mL of phosphate buffered saline. The homogenate obtained was centrifuged (3500 rpm for 30 min) and the supernatant was used to determinate some biochemical bone markers such as calcium, magnesium, inorganic phosphorus, ALP activity as well as femur oxidative status (MDA, GSH, and Nitrites). At last, histopathological analysis of heads of femur and tibia were carried on paraffin-embedded sections stained with hematoxylin-eosin.

2.4- Determination of the relative mass and femoral density

2.4.1- Determination of the relative mass of the femur and tibia

Femur and tibia relative fresh weight were calculated respectively using the formula bellow according to Akhtar *et al*. [16].

$$\text{Organ weight ratio} = \frac{\text{Femur/Tibia weight (g)}}{\text{Body weight (g)}} \times 100$$

2.4.2- Determination of femoral density

Wet femur volume was measured using a plethysmometer and its density was calculated using the formula as described by Lee *et al*. [17]:

$$\text{Femoral density} = [\text{femur wet weight (kg)} \times 1000\ (\text{kg/mm}^3)/\text{volume of femur (mm}^3)]$$

2.5- Biochemical assays

ALP activity like creatinine and IP levels, were assessed using commercial diagnostic kits LABKIT. Calcium and magnesium levels were assessed using commercial diagnostic kits Biolabo and Randox respectively.

2.6- Oxidative stress parameters assays

Malondialdehyde (MDA) and reduced glutathione (GSH) in femur homogenate were determined using methods described by Wilbur *et al*. [18] and Ellman [19] respectively while the nitrites content was determined using the method described by Green *et al*. [20].

2.7- Vaginal smears and cell differentiation

Using a micropipette, 10 µL of a 0.9% NaCl solution was introduced into the vagina of each rat and then aspirated with a bulb. The collected sample was placed on a slide and fixed in increasing baths of 50%, 70% and 80% alcohol. The slides were stained according to the method described by Papanicolaou [21].

2.8- Histopathological analysis.

After fixation of the femur and tibia in 10% buffered formalin, the organs were streamed in 3 xylene baths (10 min per bath) and then dehydrated in alcohol of croissant gradient (70%, 95%, and 100% (3 baths)). Tissues were then clarified in two xylene baths and embedded in liquid paraffin at 60°C for 4 hours. A 5 µm section of each organ was cut with a microtome, deparaffinized, and stained with hematoxylin-eosin. The microphotographies were obtained using a light microscope (Leitz wetzlar Germany 513) connected with a celestron 44421 camera linked to a computer.

2.9. Statistical analysis

Data were stated as mean ± standard error on mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test using GraphPad Prism 8.0.1. A value of $p < 0.05$ was considered statistically significant.

3- Results

3.1- Effects of P. soyauxii on relative mass of femur and tibia and on total femoral protein level

As shown by figure 1, ovariectomy resulted in a significant decrease in relative femur mass ($p < 0.01$) as well as femoral protein level ($p < 0.001$) compared to Sham control. Administration of the *P. soyauxii* extract at 200 and 300 mg/kg significantly ($p < 0.05$) increased relative femur mass compared to Ovx animals. Moreover, treatment with 200 and 300 mg/kg significantly increased femoral protein levels by $p<0.01$ and $p<0.001$, respectively, compared to Ovx animals. Besides, ovariectomy and plant extract have no effects on tibia weight.

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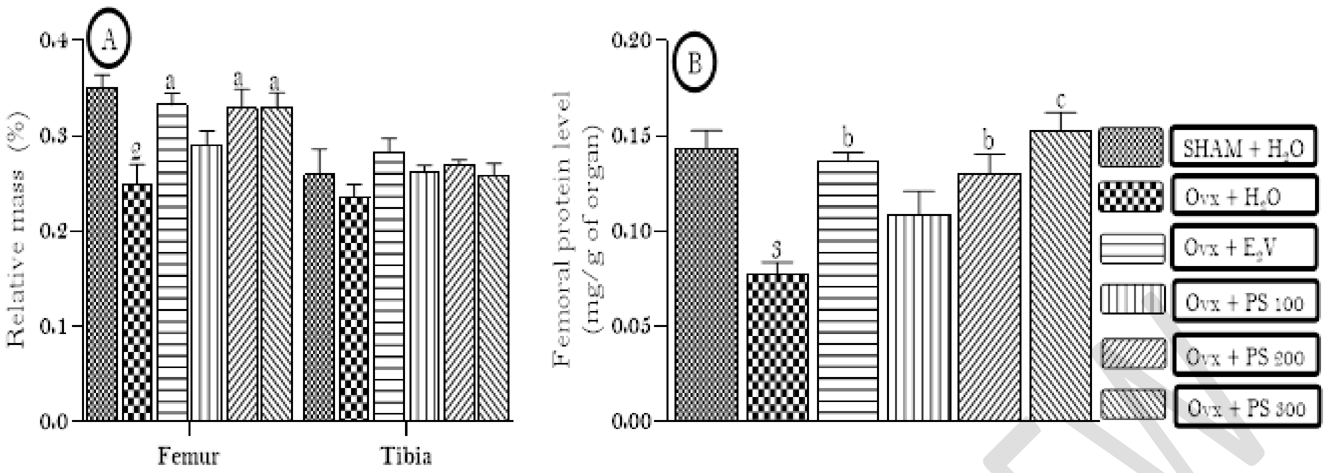

Figure 1: Effects of *Pterocarpus soyauxii* on femur and tibia relative weight (A) and femoral protein level (B). [1]p <0.05; [2]p <0.01; [3]p <0.001, significant difference compared to Sham control; [a]p <0.05; [b]p <0.01; [c]p <0.001, significant difference compared to Ovx control; **PS** = *P. soyauxii*.

3.2- Effects of P. soyauxii on femur density

The 84-day after Ovariectomy resulted in a significant decrease in femoral density ($p < 0.001$) compared to Sham control (Figure 2). Administration of *P. soyauxii* significantly increased femoral density at 200 ($p < 0.001$) and 300 ($p < 0.01$) mg/kg compared to Ovx animals.

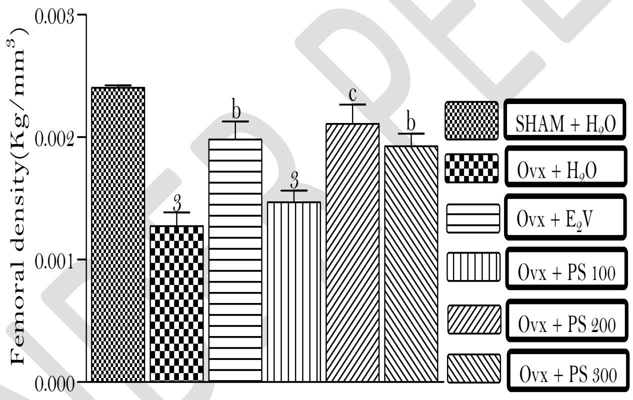

Figure 2: *Effects of P. soyauxii on femur density*.
[1]p <0.05; [2]p <0.01; [3]p <0.001, significant difference compared to Sham control; [a]p <0.05; [b]p <0.01; [c]p <0.001, significant difference compared to Ovx control; **PS** = *P. soyauxii*.

3.3- Effects of P. soyauxii heartwood on some blood elements

The effects of *P. soyauxii* aqueous extract on some blood components are shown in table 1. It shows that ovariectomy resulted in a significant ($p < 0.01$) increase in white blood cell (WBC) and a non-significant

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increase in monocytes and lymphocytes compared to Sham control. The administration *P. soyauxii* extract at 200 mg/kg reduced WBC by 86.29 % compared to Ovx control.

Parameters Groups	WBC (10^9/L)	RBC (10^{12}/L)	HGB (g/dL)	HCT (%)	LYM (10^9/L)	MO (10^9/L)
SHAM + H_2O	6.43 ± 0.73	8.26 ±0.22	13.95 ± 0.26	45.00 ± 0.77	6.79 ± 1.28	0.18 ± 0.01
Ovx + H_2O	10.14 ±0.71[2]	8.21 ± 0.22	13.57 ± 0.35	43.62 ± 0.88	8.30 ± 0.76	0.24 ± 0.01
Ovx + E_2V	6.73 ± 0.60[a]	8.73 ± 0.06	14.12 ± 0.17	46.47 ± 0.33	7.36 ± 1.36	0.18 ± 0.02
Ovx + PS 100	8.05 ± 0.70	8.81 ± 0.35	14.07±0.48	47.27 ± 1.73	7.13 ± 0.21	0.20 ± 0.02
Ovx + PS 200	8.75 ± 0.40	8.83 ± 0.08	14.65 ± 0.16	48.05 ± 0.45	7.91 ± 0.28	0.17 ± 0.02
Ovx + PS 300	9.45 ±0.79[1]	8.77 ± 0.17	14.52 ± 0.33	47.67 ± 1.35	7.60 ± 0.63	0.17 ± 0.02

Table 1: Effects of *P. soyauxii* on some blood constituents

Values represents mean ± SEM (n = 5); [1]p <0.05; [2]p <0.01, significant difference compared to Sham-operated control; [a]p <0.05, significant difference compared to Ovx control; **PS** = *P. soyauxii*, **WBC** = White blood cells*;* **LYM** = Lymphocytes*;* **RBC** = Red blood cells*;* **HGB** = Hemoglobin*;* **HCT** = Hematocrit; **MO** = Monocytes.

3.4- Effects P. soyauxii on seric, urinary, and femoral levels of calcium, phosphorus, and magnesium

Table 2 shows that a 112-day of ovariectomy induced a decrease of calcium levels in both seric and femoral homogenate ($p < 0.001$) in one hand and a significant increase ($p < 0.001$) in calcium urinary levels in other hand compared to sham control. While the administration of *P. soyauxii* extract at 100 mg/kg increased significantly ($p < 0.05$) femoral calcium level and decreased likewise the urinary one. The treatment of ovariectomized rats with the aqueous extract of *P. soyauxii* at 200 mg/kg and 300 mg/kg compared to Ovx control, increased significantly ($p < 0.05$) seric IP, levels while only the extract at 300 mg/kg increased phosphorus levels in the femur.

Table 2: Effects of aqueous extract of *P. soyauxii* heartwood on serum, urine and bone levels of calcium, phosphorus and magnesium

Parameters	**Calcium (Mmol/L)**			**Phosphorus (Mmol/L)**		**Magnesium (Mmol/L)**		
Groups	Serum	Femur	Urine	Serum	Femur	Serum	Femur	Urine
SHAM + H_2O	2.23 ± 0.05	3.18 ± 0.33	7.72 ± 0.34	2.73 ± 0.02	10.74 ± 0.25	0.45 ± 0.01	0.32 ± 0.05	2.32 ± 0.10
Ovx + H_2O	1.82 ± 0.04[3]	1.88 ± 0.23[1]	11.07 ± 0.13[3]	2.44 ± 0.05[2]	8.89 ± 0.57[1]	0.44 ± 0.00	0.20 ± 0.01	2.68 ± 0.23
Ovx + E_2V	2.13 ± 0.04[a]	2.61 ± 0.27	8.33 ± 0.28[b]	2.69 ± 0.07[b]	10.54 ± 0.20[a]	0.45 ± 0.01	0.32 ± 0.05	2.14 ± 0.10
Ovx + PS 100	1.96 ± 0.06[1]	3.09 ± 0.22[a]	9.17 ± 0.78[a]	2.63 ± 0.02	9.73 ± 0.27	0.47 ± 0.00	0.34 ± 0.04	2.25 ± 0.10
Ovx + PS 200	2.09 ± 0.01[a]	2.94 ± 0.24	8.71 ± 0.23[b]	2.73 ± 0.01[b]	10.40 ± 0.49	0.50 ± 0.02	0.25 ± 0.04	2.47 ± 0.17
Ovx + PS 300	1.93 ± 0.04[1]	2.33 ± 0.24	8.94 ± 0.48[a]	2.69 ± 0.03[b]	10.62 ± 0.13[a]	0.45 ± 0.03	0.26 ± 0.04	2.51 ± 0.49

Values represents mean ± SEM (n = 5); [1]p <0.05; [2]p <0.01; [3]p <0.001, significant difference compared to Sham-operated control; [a]p <0.05; [b]p <0.01; [c]p <0.001, significant difference compared to Ovx control; **PS** = *P. soyauxii.*

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3.5- Effects of P. soyauxii on ALP activity, creatinine levels and some osteolysis indices

The table 3 shows the effects of treatment of Ovx rats with aqueous extract of *P. soyauxii* heartwood on femoral and seric ALP activity, seric and urinary creatinine levels and some osteolysis indices. Ovariectomy resulted in a significant ($p<0.001$) decrease in femoral ALP activity and increase seric ALP activity compared with Sham-operated rats. There was also a significant ($p<0.01$) increase in the urinary calcium to urinary creatinine ratio in Ovx after 112 days. The administration of *P. soyauxii* at 200 mg/kg significantly reduced seric ALP activity ($p<0.05$) although it increased significantly ($p<0.01$) this parameter in femur compared to Ovx control.

Table 3: Effects of *P. soyauxii* on serum and bone PAL activity, urinary creatinine and some osteolysis indices

Parameters	**Creatinine (mg/dL)**		**ALP activity (IU/L)**		**$Uri-Ca^{2+}$/Uri-Crea**	**$Uri-Mg^{2+}$/Uri-Crea**
Groups	**Serum**	**Urine**	**Femur**	**Serum**		
SHAM + H_2O	1.02 ± 0.03	15.19 ± 0.19	545.66 ± 8.41	232.05 ± 6.56	0.50 ± 0.02	0.15 ± 0.01
Ovx + H_2O	0.99 ±0.02	16.10 ± 0.54	397.24 ± 15.59^{3}	185.15 ± $4,46^{3}$	0.69 ±0.02^{2}	0.16 ± 0.01
Ovx + E_2V	0.99 ± 0.02	15.40 ± 0.24	549.20 ± 16.09^{3c}	197.45 ± 2.65^{c}	0.54 ± 0.01^{a}	0.13 ± 0.01
Ovx + PS 100	0.98 ± 0.02	14.83 ± 0.55	398.39 ± 15.73^{3}	215.12 ± 4.61^{2}	0.62 ± 0.05	0.15 ± 0.03
Ovx + PS 200	0.96 ± 0.01	15.28 ± 0.10	474.71 ± 7.27^{1a}	206.55 ± 3.62^{1b}	0.57 ± 0.01	0.16 ± 0.01
Ovx + PS 300	0.95 ± 0.01	15.56 ± 0.34	454.33 ± 11.01^{2}	211.32 ± 5.06^{2a}	0.57 ± 0.02	0.16 ± 0.03

Values represents means ± SEM (n = 5); $^{1}p<0.05$; $^{2}p<0.01$; $^{3}p<0.001$, significant difference compared to Sham-operated control; $^{a}p<0.05$; $^{b}p<0.01$; $^{c}p<0.001$, significant difference compared to Ovx control; **PS** = *P. soyauxii*, **ALP** = *Alkaline phosphatase*, **Uri-Crea** = Urinary creatinine, **$Uri-Ca^{2+}$** = Urinary calcium, **$Uri-Mg^{2+}$**= Urinary Magnesium.

3.6- Effects of P. soyauxii on femoral oxidative stress status

Ovariectomy resulted in a significant decrease in GSH levels ($p<0.01$) and a significant increase in MDA ($p<0.01$) and nitrite ($p<0.001$) levels in the femur. *P. soyauxii* extract at 200 and 300 mg/kg doses compared to Ovx animals significantly increased GSH levels by ($p<0.05$) and ($p<0.01$) respectively. At all extract doses, there was a significant ($p<0.001$) decrease in femur nitrite level compared to Ovx animals. MDA level was significantly ($p<0.01$) decreased following treatment with *P. soyauxii* extract at the dose of 300 mg/kg compared to Ovx female rats (Figure 3).

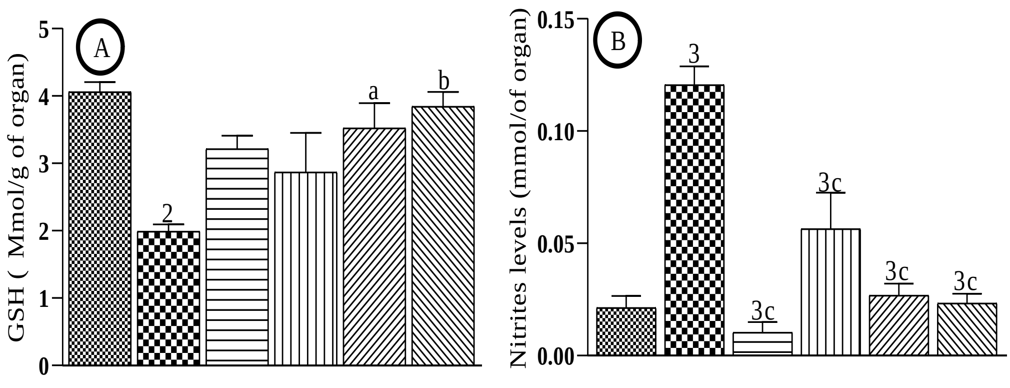

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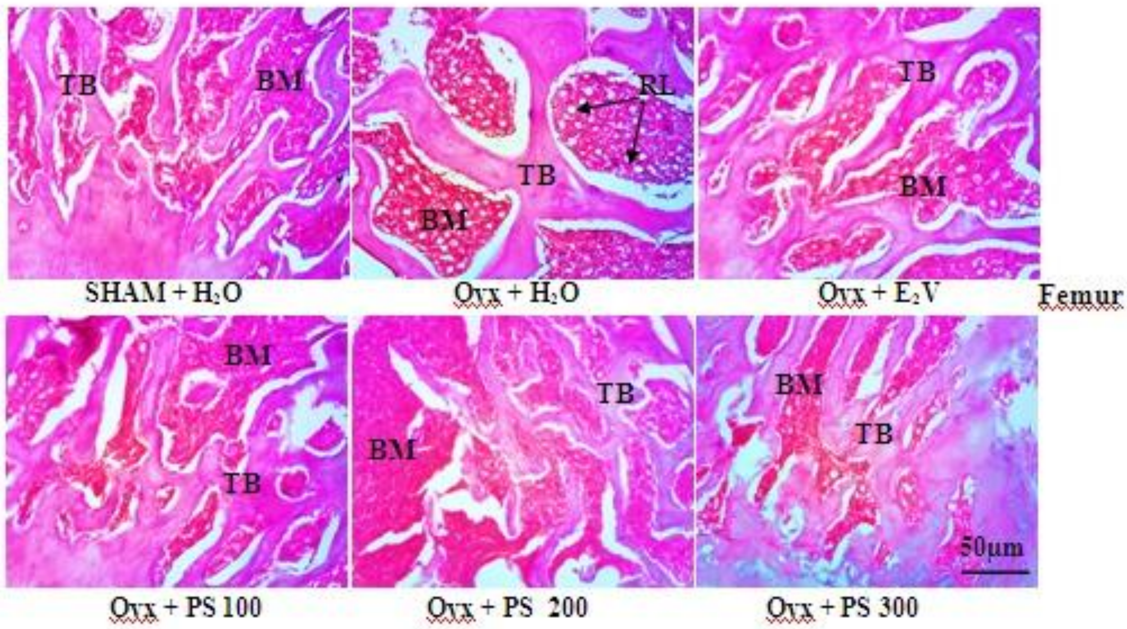
Figure 3: Effects of a 28-day treatment with *P. soyauxii* on GSH (A), nitrites (B) and MDA (C) femur levels. [1]p <0.05; [2]p <0.01; [3]p <0.001, significant difference compared to Sham-operated control; [a]p <0.05; [b]p <0.01; [c]p <0.001, significant difference compared to Ovx control; **PS** = *P. soyauxii*; **MDA** = Malondialdehyde; **GSH** = Reduced glutathione.

3.7- Effects on the microarchitecture of the femur and tibia

Figure 4 shows the effects of *P. soyauxii* extract on the microarchitecture of the femur and tibia in ovariectomized animals. Ovariectomy increased the number of resorption lacunae in the tibia and femur. Besides, it also induced tibia trabecular disorder. The oral extract corrected these alterations compared to Ovx animals at 200 and 300 mg/kg.



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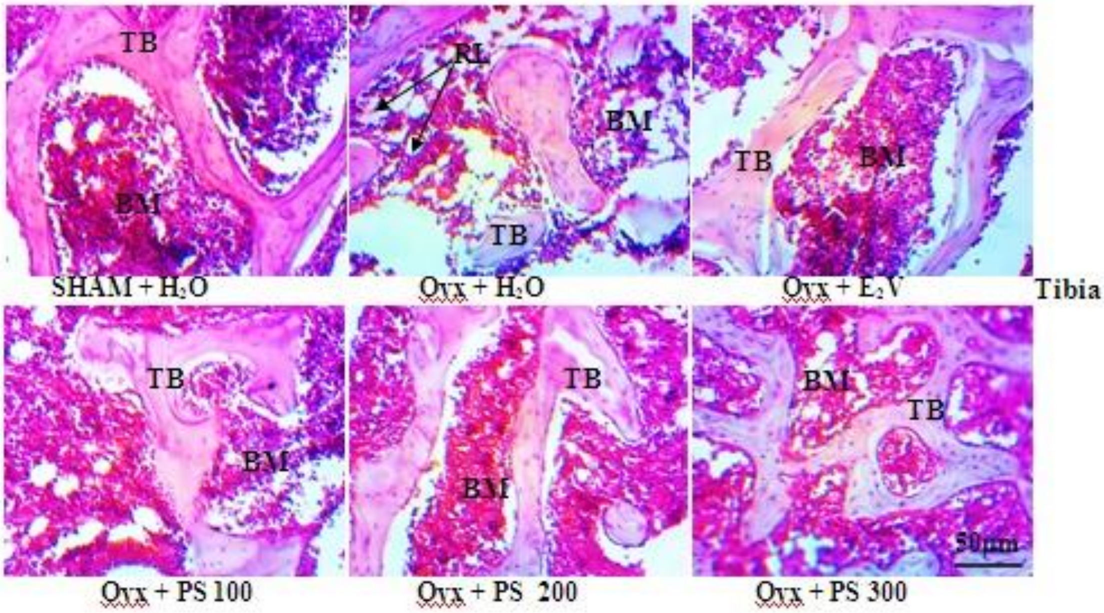

Figure 4: Microphotographs of the femur (100x, H-E) and tibia (200x, HE).
PS = *P. soyauxii*, **RL** = Resorption Lacunae, **BM** = Bone marrow, **TB** = Trabecular bone.

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4- Discussion

The potential anti-osteoporotic effects of *P. soyauxii* were evaluated in a model of postmenopausal osteoporosis induced by ovariectomy in Wistar rats. Results revealed that ovariectomy decreased significantly femur relative mass and density compared to Sham-operated animals. Indeed, estrogen is known to induce osteoclast apoptosis and osteoblast proliferation. These actions increase bone matrix synthesis, mass, and density [22-24]. In addition, Braun *et al*. [25] mentioned that high oxidative stress in hypoestrogenism was linked to osteoblast death. Furthermore, Reactive oxygen species (ROS) stimulate osteoclastogenesis [26], thus they promote bone loss. Signaling pathways are consistent in the present work with the increase of ROS like malondialdehyde (MDA) in the femur. This significant increase in MDA level could be considered as an indication of oxidative stress and cell death. Indeed, the increase in MDA level is an indicator of lipid peroxidation and thus of cell death. Some studies had the same results and showed that estrogen deficiency in ovariectomized female rats resulted in decreased relative mass and density of the femur [9, 27]. In the present study, aqueous extract of *P. soyauxii* increased femur density and relative mass in Ovx rats. This result reflects an antiresorptive activity of the plant. Qualitative and quantitative phytochemical analysis revealed flavonoids (formononetin and naringenin) in the extract of the heartwood of *P. soyauxii* [15, 28]. Naringenin is reported to possess anti-osteoporotic activity in ovariectomized rats by stimulating osteoclast apoptosis and osteoblastic proliferation [29]. Besides, formononetin preserves femoral mass and density through its ability to bind to estrogen receptors located on the bone and exert antioxidant activity to increase bone mineral density [30-32]. In addition, LCMS analysis revealed that *P. soyauxii* aqueous extract possesses linoleic acid [10]; a molecule capable of preventing bone loss by stimulating osteoprogenin (OGP) expression and inhibiting RANKL expression in ovariectomized animals according to Rahman *et al.* [8].

Measurement of bone metabolism markers plays an important role in the diagnosis and treatment of osteoporosis [33]. Bone loss in many studies is evidenced by increased calcium, phosphorus, alkaline phosphatase (ALP) activity in blood and urine, and urinary calcium/creatinine ratio [34]. Other work also explains the development of osteoporosis by a decrease in bone concentrations of calcium, phosphorus, and ALP activity [9,35]. Ovariectomy in the same sense varied seric, femoral and urinary biochemical markers of bone metabolism evaluated in the present work. Indeed, a study reported that ovariectomy modified biochemical markers of bone metabolism [9], by the way, Hewitt *et al*. [36] show that ALP modulates osteoblastic activity and is associated with bone mineralization. Furthermore, hypoestrogenism is related to bone demineralization by Braun *et al*. [25] and the results of the present study corroborate this point. Indeed, 112-day Ovx animals presented a high osteolysis index (urinary calcium/ urinary creatinine). *P. soyauxii* extract, as well as estradiol valerate, reestablished normal values of biochemical markers of bone metabolism compared to Ovx rats. Phytoestrogens are known to possess positive action on bone mineralization and osteoblastic differentiation as well as inhibition of osteoclastic activity [37-38]. Linoleic acid, a phytoestrogen identified in the heartwood aqueous extract [10] is probably responsible for osteoprotective activity by decreasing the concentration of serum biochemical markers of bone resorption in ovariectomized animals [9].

Inflammation may contribute to osteoporosis [39]. The results of the present study show in Ovx animals a significant elevation of bone nitrite; a surrogate for nitrite oxide (NO) in ovariectomized animal. Analysis of hematological parameters also revealed an increase in white blood cells count, in Ovx rats compared to sham-operated rats. Cuzzocrea [40] reported the same results and suggested that the nitrites produced following oophorectomy would come from a macrophagic activity and are a sign of bone inflammation. NO appears to exert biphasic effects by affecting the bone formation and resorption processes in osteoblasts and osteoclasts. Bone formation increased while bone resorption is suppressed at low NO concentration [41]. *P. soyauxii* extract at all doses decreased femoral nitrite level and non-significantly decreased the white blood cell level. Indeed, the work of Saliu *et al*. [42] highlighted the richness of *P. soyauxii* in kaempferol and quercetin. Kaempferol and quercetin potentially protect bone through their anti-inflammatory properties on osteoblastic cells by inhibiting nuclear translocation and NF-κB activation [43-44]. Linoleic acid a molecule reported in *P. soyauxii* extract [10] is known to inhibit the production of inflammatory cytokines that are messengers for osteoclast recruitment and differentiation [45]; thus, explaining the anti-osteoporotic activity of *P. soyauxii* through the anti-inflammatory pathway.

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In addition to inflammatory processes, oxidative stress is an important factor in the pathogenesis of osteoporosis, which is manifested in many studies by microarchitectural alterations of the femur and tibia. In the present work, analysis of oxidative status markers revealed the increase in MDA level and the decrease in GSH level in the femur of Ovx animals compared to sham-operated control; reflecting femoral oxidative stress in Ovx rats. This oxidative stress was correlated with numerous resorption lacunae on histological sections of femur and tibia. This oxidative stress in bones is thought to be related to post-ovariectomy estrogen deficiency. Many studies reported that ovariectomy caused oxidative bone stress in rats marked by an increase in MDA levels and a decrease in GSH levels but also the appearance of resorption lacunae on histological sections of the femur [9]. Indeed, ROS formed under hypo-estrogenic conditions stimulate osteoclast formation and activity, thus they decreased osteoblastic function and osteoblast recruitment, and collagen synthesis [46-47]. The administration of *P. soyauxii* extract reestablished the homeostasis of oxidative parameters in femur and prevented microarchitectural alterations in femur and tibia. Pterostilbenes contain in *P. soyauxii* extract reported to possess antioxidant activity by reducing the expression of NADPH oxidase [10,48]. This activity would explain the antioxidant effects of *P. soyauxii* on bone.

Conclusion

Hypoestrogenism induced by ovariectomy resulted in disorders of bone metabolism and microarchitecture. *P. soyauxii* extract fixed these alterations. The osteoprotective effects of *P. soyauxii* would be related to its antioxidant, estrogenic and probably anti-inflammatory activities. It would be necessary to evaluate *in vitro*, the osteoprotective signaling pathways of *P. soyauxii*.

References

1. Choi MH, Yang JH, Seo JS, Kim YJ, Kang SW. Prevalence and diagnosis experience of osteoporosis in postmenopausal women over 50: Focusing on socioeconomic factors. PLoS ONE. 2021;16(3): e0248020.
2. Graham LS, Parahami F, Tintut Y, Kitchen CM, Demer LL, Effors RB. Oxidized lipids enhance RANKL production by T lymphocytes: Implications for lipid-induced bone loss. Clin Immunol. 2009; 133(2):265-275.
3. Jakob F, Seefried L, Ebert R. Pathophysiology of bone metabolism. (2008): Internist. 2008; 49:1159-1169.
4. Michael A, Clynes, Nicholas C, Harvey, Elizabeth M, Curtis, Nicholas R. Fuggle, Elaine M. Dennison, and Cyrus Cooper. The epidemiology of osteoporosis. Br. Med. Bull. 2020; 133:105-117.
5. Kapur P, Harry H, Wuttke W, Pereira BMJ, Seidlova-Wuttke D. Evaluation of the antiosteoporotic potential of *Tinospora Cordifolia* in female rats. Maturitas. 2008; 20;59(4):329-38.
6. WHO. Traditional medicine: growing needs and potential. WHO policy perspectives on medicines, World Health Organization Geneva 2. 2002; 1-6.

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7. Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestinin healthy postmenopausal women principal results from the women's health initiative randomized controlled trial. JAMA. 2002; 288:321-333.
8. Rahman M, Fernandes G, Williams P. Conjugated linoleic acid prevents ovariectomy-induced bone loss in mice by modulating both osteoclastogenesis and osteoblastogenesis. Lipids. 2014; 49(3): 211-224.
9. Oumarou M, Zingue S, Bakam B, Ateba S, Harquin Foyet S, Fritz T, Tchaptchet M, Njamen D. *Lannea acida* A. Rich. (Anacardiaceae) ethanol extract exhibits estrogenic effects and prevents bone loss in an ovariectomized rat model of osteoporosis. eCAM. 2017; Article ID 7829059, 16 pages.
10. Mengue NYS, Owona PE, Noubom M, Mbock MA, Mbolang NL, Ngoungouré MC, Ngapout FR, Bidingha GR, Kahou T RB, Bilanda DC, Kamtchouing P, Dzeufiet DPD. Estrogenic and antioxidant activities of *Pterocarpus soyauxii* heartwood aqueous extract in bilateral oophorectomized *Wistar* rat. eCAM. 2021 ; Article ID 6759000, 18 pages.
11. Betti J, Lejoly J. Contribution to the knowledge of medicinal plants of the Dja Biosphere Reserve, Cameroon: Plants used for treating jaundice. J. Med. Plant Res. 2009; 3: 1056-1065.
12. Betti J. An ethnobotanical study of medicinal plants among the Baka pygmies in the Dja biosphere reserve, Cameroon. Afr. Study Monogr. 2004; 25: 1-27.
13. Moronkola D, Oladapo T, Adegbenro F, Ogunbanjo D, Olayinka K. Chemical constituents in essential oils of *Pterocarpus soyauxii* leaf, leaf stalk and stem bark. Drug Discov Ther*;* 2019; 9: 206-211.
14. Mpondo C, Ngene J, Mpounze S, Etame L, Boumsong P, Yinyang J, Dibong S. Connaissances et usages traditionnels des plantes médicinales du département du haut Nyong. J. Appl. Biosci. 2017 ; 113 : 11229-11245.
15. Saslis-Lagoudakis H, Klitgaard B, Forest F, Francis L, Savolainen V, Elizabeth M, Hawkins J. (2011). the use of phylogeny to interpret cross-cultural patterns in plant use and guide medicinal plant discovery: An Example from Pterocarpus (Leguminosae), PLoS ONE; 6:7e22275.
16. Akhtar NMK, Srivastava, Raizada RB. Assessment of chlorpyrifos toxicity on certain organs in rat, *Rattus norvegicus*. J. Environ. Biol. 2009; 30(6):1047-1053, 2009.
17. Lee YB, Lee HJ, Kim KS. Evaluation of the preventive effect of isoflavone extract on bone loss in ovariectomized rats. Biosci. Biotechnol. Biochem. 2004; 68(5):1040–1045.
18. Wilbur KM, Bernheim F, Shapiro OW. The Thiobarbituric acid reagent as a test for the oxidation of unsaturated fatty acids by various agents. Arch. Biochem. Biophys. 1949; 24(2): 305-313.
19. Ellman GL. Tissue sulfhydryl group. Arch. Biochem. Biophys. 1959; 82(1):70-77.
20. Green LC, Wagner DA, Godowsky J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. Anal. Biochem. 1982; 126(1): 131-138.

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21. Fortier JF, Hould R. Histotechnologie : Théories et procédés, CCDMD edition edition, 2003.
22. Turner RT. Mice, estrogen, and postmenopausal osteoporosis. J Bone Miner Res. 1999; 14(2): 187-191.
23. Turner RT, Glenn KW, Kathleen SH. Differential effects of androgens on cortical bone histomorphometry in gonadectomized male and female rats. 1990. J Orthop Res; 8(4): 612-617.
24. Kameda T. Mano H, Yuasa T, Mori Y, Miyazawa K, Shiokawa M, Nakamaru Y, Hiroi E, Hiura K, Kameda A, Yang NN, Hakeda Y, Kumegawa M. Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. J Exp Med. 1997; 186(4): 489-495.
25. Braun KF, Ehnert S, Freude T, Egaña JT, Schenck TL, Buchholz A, Schmitt A, Siebenlist S, Schyschka L, Neumaier M, Stöckle U, Nussler AK. Quercetin protects primary human osteoblasts exposed to cigarette smoke through activation of the antioxidative enzymes HO-1 and SOD-1. Sci. World J. 2011 11:2348-57.
26. Kim HJ, Chang EJ, Kim HM, Lee SB, Kim HD, Su KG, Kim HH. Antioxidant alpha-lipoic acid inhibits osteoclast differentiation by reducing nuclear factor kappa B DNA binding and prevents in vivo bone resorption induced by receptor activator of nuclear factor-kappa B ligand and tumor necrosis factor alpha. Free Radic Biol Med. 2006; 1;40(9):1483-93.
27. Patsaki A, Tchoumtchoua J, Passali C, Lelovas P, Kourkoulis S, Papaioannou N, Mbanya JC, Njamen D, Mitakou S, Halabalaki M, Dontas I. The Protective Effect of *Amphimas pterocarpoides* plant extract on bone mineral density and strength in estrogen deficient ovariectomized wistar rats. Med Aromat Plants. 2016; 5(5):1-7.
28. Barend C, Bezuidenhoudt E, Vincent B, Daneel F. Flavonoid analogues from Pterocarpus species. Phytochemistry. 1987; 26: 531- 535.
29. Zhu Z, Wenjing X, Yanyan L, Zaiou Z, Wei Z. Effect of naringin treatment on postmenopausal osteoporosis in ovariectomized rats: A meta-analysis and systematic review. eCAM. 2021; Article ID 6016874, 8 pages.
30. Kaczmarczyk-Sedlak I, Weronika W, Maria Z, Ewa O, Joanna T, Agata S. Effect of formononetin on mechanical properties and chemical composition of bones in rats with ovariectomy-induced osteoporosis. eCAM. 2013; Article ID 457052, 10 pages.
31. Ha H, Lee HY, Lee JH. Formononetin prevents ovariectomy-induced bone loss in rats. Arch. Pharm. Res. 2010; 33(4):625-632.
32. Mu H, Bai YH, Wang ST, Zhu ZM, Zhang YW. Research on antioxidant effects and estrogenic effect of formononetin from *Trifolium* pretense (red clover). Phytomedicine. 2009; 16(4):314-319.
33. Allende-Vigo MZ. The use of biochemical markers of bone turnover in osteoporosis. P. R. Health Sci. J. 2007; 26(2): 91-95.

UNDER PEER REVIEW

34. Alavizadeh H, Hosseinzadeh H. Bioactivity assessment and toxicity of crocin: a comprehensive review. Food Chem. Toxicol. 2014; 64:65-80.
35. Chitme HR, Muchandi IS, Burli SC. Effect of *Asparagus Racemosus* Willd root extract on ovariectomized rats. Open Nat. Prod. J; 2009, 2, 16-23.
36. Hewitt SC, Deroo BJ, Hansen K, Collins J, Grissom S, Afshari CA, Kenneth S. Estrogen receptor dependent genomic responses in the uterus mirror the biphasic physiological response to estrogen. J. Mol. Endocrinol, 2003; 17(10):2070-2083.
37. Rassi CM, Lieberherr M, Chaumaz G, Pointillart A, Cournot G. Down-regulation of osteoclast differentiation by daidzein via caspase 3. J Bone Miner Res. 2002; 17: 630-638.
38. Vergne S, Bennetau-Pelissero C, Lamothe V, Chantre P, Potier M, Asselineau J, Perez P, Durand M, Moore N, Sauvant P. Higher bioavailability of isoflavones after a single ingestion of a soya-based supplement than a soya-based food in young healthy males. Br J Nutr. 2008; 99:333-344.
39. Yun AJ, Lee PY. Maldaptation of the link between inflammation and bone turnover may be a key determinant of osteoporosis. Med Hypotheses. 2004; 63(3):532-537.
40. Cuzzocrea S, Emanuela M, Laura D, Tiziana G, Rosanna DP, Zaira R, Elisabetta V, Achille PC, Fons AJV, Domenico P, Adriana M. Inducible nitric oxide synthase mediates bone loss in ovariectomized mice. Endocrinology. 2003; 144(3):1098-1107.
41. Wimalawansa SJ. Nitric oxide and bone. Ann N Y Acad Sci. 2010; 1192:391-403.
42. Saliu J, Schetinger M, Ganiyu O, Olasunkanmi S, João B, Roberta S, Jessie G, Naiara S, Fabiano C,Vera M, Aline B. Effect of dietary supplementation of Padauk (*Pterocarpus soyauxii*) leaf on high fat diet/streptozotocin induced diabetes in rats brain and platelets. Biomed. Pharmacother. 2016; 84: 1194-1201.
43. Pang J, Dennis AR, Su H, Nigar F, Dhirendra PS, Jose RR, Naibedya C. Differential activity of kaempferol and quercetin in attenuating tumor necrosis factor receptor family signaling in bone cells. Biochem Pharmacol. 2006; 71:818-826.
44. Zhu J, Tang H, Zhang Z, Yong Z, Chengfeng Q, Ling Z, Pinge H, Feng L. Kaempferol slows intervertebral disc degeneration by modifying LPS-induced osteogenesis/adipo-genesis imbalance and inflammation response in BMSCs. Int Immunopharmacol. 2017; 43:236-242.
45. Barbour KE, Boudreau R, Danielson ME, Youk AO, Wactawski-Wende J, Greep NC, LaCroix AZ, Jackson RD, Wallace RB, Bauer DC, Allison MA, Cauley JA. Inflammatory markers and the risk of hip fracture: The Women's Health Initiative. J Bone Miner Res. 2012; 27:1167-1176.
46. Aiwo SA, Chaekyun K. Reactive oxygen species in osteoclast differentiation and possible pharmaceutical targets of ROS-mediated osteoclast diseases. Int. J. Mol. Sci. 2019; 20: 1-16.
47. Huan Q, Wenwen Z, Yang J, Haoyi Z, Hao Z, Jingyu J, Qiu L, Xiuping C, Xia G and Yantao H. Aqueous extract of *Salvia miltiorrhiza* bunge-radix puerariae herb pair attenuates osteoporosis in

UNDER PEER REVIEW

ovariectomized rats through suppressing osteoclast differentiation. Front. Pharmacol. 2021; 11: 581049.

48. Zhang Y, Zhang Y. Pterostilbene, a novel natural plant conduct, inhibits high fat induced atherosclerosis inflammation via NF kappa B signaling pathway in Toll like receptor 5 (TLR5) deficient mice. Biomed. Pharmacother. 2016; 81(1):345-355.

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