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Comparative effect of *Pennisetum glaucum* and alendronate on biochemical and histological analyses of lumbar vertebrae and liver for rats fed cafeteria diet associated with glucocorticoid therapy

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Abstract

Glucocorticoid therapy causes serious side effects, including osteoporosis, growth delay, bone fragility, and increasing bone fractures. The purpose of this study is to evaluate the effect of aqueous extract of *Pennisetum glaucum* (AEPG) and alendronate (ALN) on lumbar vertebrae in rats fed cafeteria (CAF) diet associated with methylprednisolone (MPN), a glucocorticoid widly used in therapy. Thirty six Wistar rats were randomized into six groups of six animals each: control group received standard diet for rats; CAF group received cafeteria diet; CAF+MPN group received CAF+MPN (9 mg/kg body weight intraperitoneal); CAF+AEPG group received CAF+AEPG (250 mg/kg); CAF+MPN+AEPG group received (CAF, 9 mg/kg, 250 mg/kg respectively); CAF+MPN+ALN group (CAF, 9 mg/kg, 2 mg/kg respectively). After six weeks of treatment, animals were weighted and sacrificed. Lumber (L_1) mineral content was assessed. Lumbar (L_{2-3}) and liver serial sections of 5 µm thicknesses were cut for pathology. Our data showed that calcium and phosphorus content decreased in MPN groups. AEPG, CAF and ALN seem to correct glucocorticoid therapy phosphocalcic disorders. We found significant histopathological alterations signing a hepato-steatosis in both CAF and CAF+MPN groups; alterations healed by AEPG. The groups receiving CAF and MPN show bone loss, and decreased lumbar trabecular bone. In our research CAF+MPN+ALN and CAF+MPN+AEPG groups showed a greater improvement of lumbar trabecular bone. AEPG demonstrated, as well as ALN, a beneficial effect on bone fragility induced by methylprednisolone treatment. *Pennisetum glaucum*, an ethnopharmacological plant, may be used or associated in osteoporosis treatment and bone fragility prevention.

1. Introduction

Bone is a multicomposite material that consists of cells, extracellular organic components (collagen and non-collagenous matrix protein) and nonorganic components (calcium hydroxyapatite) [1]. Glucocorticoids are important in the therapy of asthma, chronic diseases, inflammatory and rheumatoid arthritis [2,3]. It is generally demonstrated that glucocorticoid treatment causes many complications. It has been known that glucocorticoid (GC) therapy increase risk for developing osteoporosis [4]. Osteoporosis is defined by the World Health organization (WHO) as a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk, it is a major health problem in the worldwide [5]. Risk of vertebral fracture caused by glucocorticoid-induced osteoporosis depends with the therapy dose [6]. However glucocorticoid therapy is not the only contributor of bone disorders. Other risk factors can be implicated including imbalanced foods. Thus, high fat diet has a great risk for bone fragility fractures [7-9].

Bisphosphonates (BP) are widely used in therapy for the prevention and treatment of glucocorticoid-induced bone loss at the lumbar spine [10]. Several studies showed that BP can preserve bone mass and reduce the incidence of vertebral fractures for rheumatic patients [11]. The combination treatment GC and BP decreases risk of vertebral fractures [10]. It is widely reported that alendronate, a BP is used to prevent glucocorticoid-induced osteoporosis [12].
In folk medicine various plants extracts has been found useful for their therapeutic effect on traumatisms and bone fractures. Pearl millet (*Pennisetum glaucum* L.) belongs to the family of Poaceae. It is a road nutritionally and pharmacologically vegetable grows in several countries. In Moroccan ethnopharmacology, pearl millet is used as treatment for trauma, pain and bone fractures, but it has not been evaluated scientifically. The purpose of this study is to evaluate the effect of aqueous extract of *Pennisetum glaucum* (AEPG) and alendronate (ALN) on lumbar vertebrae in rats fed high calorie diet associated with methylprednisolone (MPN).

2. Materials and methods

2.1. Animals
The 6-week-old Wistar rats (50 – 60 g) were used for these experiments respecting all the Rabat Medical Pharmacy School. They were housed three per plastic cages (42 × 26 × 18 cm) in a room at 25° C controlled temperature, humidity relative of 55% and having in 12 h light/ 12 h dark cycle. All animals were provided with water and standard diet ad libitum and allowed to acclimate for one week. All procedures performed throughout the experiment conformed to the guidelines and the Council instructions about the protection of living animal used in scientific investigations.

2.2. Fat diet: Cafeteria diet
The cafeteria diet (CAF) selected, providing modified versions of Harris (1993) high fat diet [13]. It consisted of 3 variants: (i) condensed milk + bread + peanuts + pellet chow (4:1:4:1), chocolate + biscuits + dried coconut + pellet chow (3:2:4:1), and cheese + boiled potatoes + beef tallow + pellet chow (4:2:4:1). The different variants were presented on alternate days throughout the treatment period [14].

2.3. *Sample preparation plant*
Whole grains of pearl millet (*Pennisetum glaucum* L.) were obtained from Rabat herbalists (Morocco). The grains identified by the botanic department of the National Scientific Institute, dried at room temperature and grounded to obtain a fine powder using an electric blender. For the aqueous extract of *Pennisetum glaucum* (AEPG), 1 kg of fine powder was mixed with distilled water (3 volumes) in a glass jar and left for 2 days at room temperature in an orbital digital agitator (Rotatest 560VIT. 15-300 T/MIN). The solvent was filtered through Whatman pleated filter paper. The filtrate was concentrated under reduced pressure using a rotary vacuum evaporator [15] and stored at 4°C until further used.

2.4. *Experimental protocol*
The animals were randomly assigned and divided into six groups (n = 6) for six weeks. All the experiments were conducted between 10:00 and 12:00 hour, and various products were tested as follows: control group fed on standard diet, cafeteria group nourished with cafeteria diet (CAF), CAF+MPN group received CAF + MPN (9 mg/kg b.w, intraperitoneal (ip)), CAF+AEPG group received CAF+AEPG (250 mg/kg b.w, per os (po)), CAF+MPN+AEPG group received[CAF, MPN (9 mg/kg ip), AEPG (250 mg/kg b.w, po)], CAF+MPN+ALN group received [CAF+ MPN (9 mg/kg ip) + ALN (2 mg/kg b.w, po) respectively].
The AEPG was suspended in distilled water and administered orally for 5 days/week at a constant volume (250 mg/kg b.w.) [15]. At the end of the 6-week experimentation, all rats were weighed and anesthetized using an overdose of diethyl ether then sacrificed.

2.5. *Body weight and macroscopical parameters*
We weighted the animals at the initial of experimentation and after six weeks of treatment. Then after sacrifice, all lumbar vertebrae were removed and cleaned of all soft tissue. The bones were immediately weighted, with an electronic balance to receive wet bone weight (WW) and their length and width were measured by calliper [16], and fixed in 10% neutral buffered formalin.

2.6. *Lumbar mineral analysis*
The lumbar (L1) was cleaned of muscular and conjunctive tissues and weighted to determine the wet bone weight (WW). Lumbar (L_1) was placed in an incubator (70°C) for 48 hours, and weighted to determinate the

dried weight (DW) for water content percentage (% water composition = [(WW-DW)/WW]×100). After recording of dry weights, the bones were digested by placing them in solution of 5 mL concentrated nitric acid (NHO_3), 3 mL 70% perchloric acid ($HClO_4$) and 2 mL 35% hydrogen peroxide and microwaving at 180 PSI and 180°C [17]. Mineral concentrations were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Horiba, JobinYvon Ultima 2, French).

2.7. *Histological evaluation*

Lumbar vertebrae (L_2-L_3) and liver were fixed in 10 % neutral-buffered formalin for assess histology tissues. After bone decalcification in 5% nitric acid for 27 days, the samples were dehydrated by graded series of alcohols (Schrlau, Scharlab S.L, Spain) from 50%, 70%, 80%, 90%, 95%, 100%. The specimens were cleared and impregnate in Toluene (Prolabo, Analo R Normapur, VWR International SAS, French). Following dehydration, the bone and liver samples were embedded in paraffin and 5 µm thick slices were taken from the length, and hemalun hetoxylin, in addition to the coloring of Manson's Trichrome. The slides were observed by light microscope (Leica L 2500 Microsystems, Germany) [18] at staining (200× magnification). We used the LAS EZ software version 3.0 to explore the pictures.

2.8. *Scanning electron microscopy*

Bone structure was evaluated by scanning electronic microscopy. After fixation and un decalcified lumbar (L_4) samples were dehydrated through graded series (70%, 90%, 95%, 100%) of alcohol [19], and then evaluated in environment SEM operating (Philips XL series, FEI Quanta 200) at 30 kV, images (500× magnifications) and 200.0 µm.

2.9. Statistical analysis

Data was presented as the mean ± SEM for ten rats per group (n = 6). Multiple comparisons were analyzed between groups using one way analysis of variance (ANOVA) and Bonferroni's multiple comparison tests. Significance was established at $p<0.05$ comparing all treatment groups to control. Data analyses were performed using Graph Pad Prism, version 6.

3. Results and discussion

3.1. *Body weight and macroscopical parameters*

The present study is carried out to evaluate the extract aqueous of pearl millet effect compared with alendronate in rats fed on cafeteria diet associated with methylprednisolone. Glucocorticoids remain an effective therapeutic option commonly used by clinicians and researches in the treatments of many inflammatory and autoimmune diseases [1], but sides' effects are observed with this therapy.

Table 1 contains the main values of the initial and final body weight (g) of rats after six weeks of treatment;

Table 1: Effects of PG and ALN on initial and final body weight in experimental rats. Rats were weighed three times week for evaluate the dose administration throughout the 6 weeks course of the treatment.

Groups	Initial body weight (g)	Final body weight (g)
Control	57.00	117.12
Untreated CAF group	57.47	171.40***
CAF+MPN (9 mg/kg b.w)	57.51	129.47
CAF+AEPG (250 mg/kg b.w)	57.29	159.88**
CAF+MPN (9 mg/kg b.w) +AEPG (250 mg/kg b.w)	57.10	129.67
CAF+MPN (9 mg/kg b.w) + ALN (2 mg/kg b.w)	57.77	116.48

*$p<0.05$ control group

Results showed no significant differences in initial body weight between groups. We revealed that the body mass progressively increased in rats fed cafeteria diet ($p<0.001$) and CAF+AEPG ($p<0.01$) compared to the control group, and decreased in MPN groups compared to CAF group. This last result is confirmed by some authors who reported that methylprednisolone attenuated the increase in body mass [20].

The lumbar vertebrae (L_{1-5}) weight and length; and also lumbar (L_1) water content didn't significant increase in CAF and CAF+AEPG groups compared to other groups (figure 1, A, C, and D). Our data showed that the lumbar weight moderately decreased in groups treated with methylprednisolone compared to CAF group (figure 1, graph A). It has been well reported that steroids including GC, suppressed growth by inhibition of growth hormone or somatomedin and affect calcium and phosphorus metabolism [21].

In this study, we assessed the effect of AEPG and ALN on the hepatic tissues for rats fed cafeteria diet and administered intramuscular methylprednisolone during thirty six weeks of treatments. We measured the liver weight of all animals. The MPN therapy significantly decrease the liver weight compared to control and CAF groups (graph B). The liver weight significantly diminished also in CAF+MPN+ALN group compared to the CAF+MPN group ($p<0.5$)

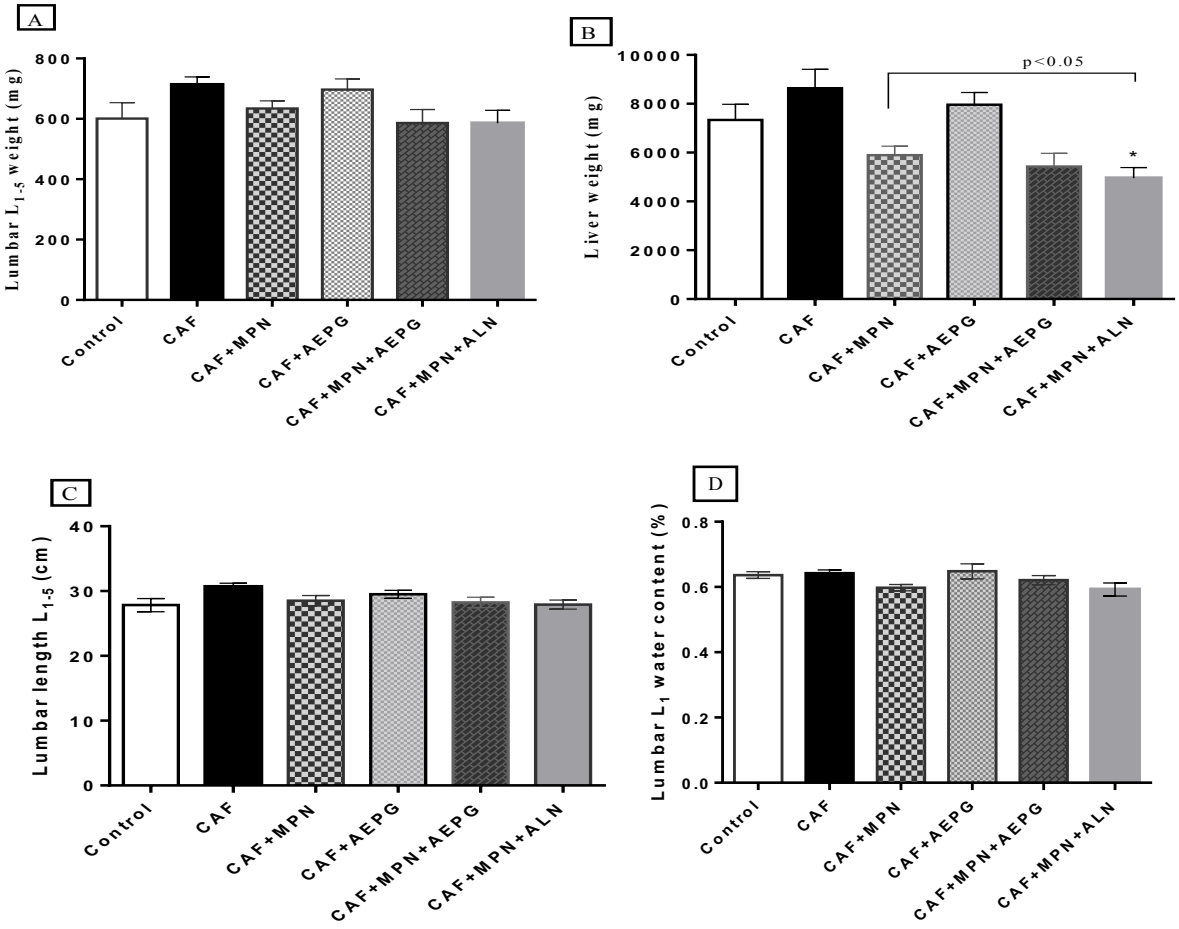

Figure 1: Effects of AEPG and ALN on lumbar vertebrae weight (A), liver weight (B), lumbar vertebrae length (C) and lumbar vertebrae water content (D) exposed to cafeteria diet, 9 mg/kg of methylprednisolone, 250 mg/kg of AEPG and 2mg/kg of alendronate and the control rats. CAF: cafeteria diet, GC: glucocorticoid, PG: *Pennisetum glaucum*, ALN: alendronate.

3.2. *Mineral concentrations*

Bone strength is characterized by degree of bone mineralization [22]. Bone wet weight and calcium content decreased in ovariectomy which decrease estrogenic activity and affect the bone mass and constituency calcium and zinc concentration [23]. Similar results can be observed in bone tissue in rats treated by GC. In this study (table 2), we observed that the calcium content decreased in MPN groups. The decrease in the calcium content in MPN groups can be explained by deceased intestinal calcium absorption, and increased renal calcium clearance [24]. We also noted that calcium and phosphorus content of lumbar vertebrae increased in groups treated with CAF+AEPG and CAF compared to groups treated with MPN (table 2). Nevertheless, we observed no significant differences increase in CAF+MPN+AEPG and CAF+MPN+ALN groups compared to the control group. Our data showed that cafeteria diet increased calcium and phosphorus content in lumbar and seem to attenuate corticosteroid effect on phosphocalcic disorders in this present study.

In addition, we didn't note significant changes in other minerals content, except manganese which shows a significant decrease in both CAF+MPN+AEPG and CAF+MPN+ALN groups compared to control group.

Table 2: Bone mineral composition in lumbar vertebrae following 9 mg/kg methylprednisolone, 250 mg/kg of AEPG, and 2mg/kg of ALN treatment for 6 weeks (mean ± SEM)

Parameters	Control	CAF	CAF+MPN	CAF+AEPG	CAF+MPN+AEPG	CAF+MPN+ALN
Ca (%)	15.47 ±0.12	42.02±9.93	14.23±0.22	36.89±15.26	15.45±0.45	15.93±0.73
Mg (%)	0.38±0.00	0.67±0.17	0.34±0.00[a]	0.73±0.27	0.34±0.01	0.32±0.01[ae]
P (%)	13.25±0.67	21.64±10.02	12.13±0.68	26.78±10.45	12.96±0.24[a]	12.34±0.38
Fe (%)	0.02±0.01	0.03±0.00	0.04±0.01	0.04±0.01	0.016±0.00[b]	0.02±0.00[be]
Zn (%)	0.40±0.00	0.03±0.01	0.02±0.00[a]	0.05±0.01	0.05±0.02	0.03±0.00[a]
Mn (≤mg/L)	3.12±0.09	3.23±0.22	3.62±0.12	3.46±0.18	4.41±0.09[a]	4.36±0.08[abd]
Ca/P (%)	1.18±0.50	0.98±0.24	1.33±0.13	1.19±0.08	2.32±0.73	1.29±0.02

Data are expressed as mean ± SEM. An ANOVA with Bonferroni's test. Control, CAF: cafeteria diet, CAF+MPN: cafeteria + glucocorticoid, CAF+AEPG: cafeteria + *Pennisetum glaucum*, CAF+MPN+AEPG: cafeteria diet + glucocorticoid + *Pennisetum glaucum*, CAF+MPN+ALN: cafeteria diet + *Pennisetum glaucum* + alendronate. Ca: calcium, Mg: magnesium, P: phosphorus, Fe: iron, Zn: zinc, Mn: manganese, Ca/P: ratio calcium phosphate.

[a]p<0.05vs control group, [b]p<0.05 vs CAF group, [c]p<0.05 vs CAF + MPN group, [d] p<0.05 vs CAF+AEPG group, ep<0.05 vs CAF+MPN+AEPG group.

[a]p<0.01, [a]p<0.05 vs CAF+AEPG, CAF+MPN+ALN (Mg) respectively. [e]p<0.01 vs CAF+MPN+ALN, (Mg).
[a]p<0.001vs CAF+AEPG+AEPG, (P).
[b]p<0.05, [b]p<0.05 vs CAF+MPN+AEPG, CAF+MPN+ALN, (Fe) respectively, [e]p<0.05 vs CAF+MPN+ALN, (Fe)
[a]p<0.01, [a]p<0.05 vs CAF+MPN, CAF+MPN+ALN, (Zn) respectively.
[a]p<0.01vs CAF+MPN+AEPG, CAF+MPN+ALN (Mn) respectively, [b]p<0.01 vs CAF+MPN+ALN (Mn), [d]p<0.05 vs CAF+MPN+ALN (Mn).

3.3. *Histological evaluation*

Several authors reported that glucocorticoid has been implicated in the pathogenesis of nonalcoholic fatty liver disease [25, 26]. We found significant histopathological alterations associated with several fat vacuoles (figure 2, b1 and c1) observed in both CAF and CAF+MPN groups compared with other groups which indicate the development of hepatosteatosis. We also observed healthy livers with no steatosis and histologically normal hepatic cords and parenchyma in control group, AEPG and ALN groups. This data suggests that AEPG was benefic to correct the hepatic cells and decrease microvesicular steatosis. Brante et al reported that cafeteria diet caused the most extensive steatosis and distorted cord architecture of liver [27]. The liver disease is part of the potential causes for secondary osteoporosis [28]. Bone disease management for liver patients is addressed to reduce or avoid the risk factors for osteoporosis and fracture [29].

Several animal studies focused on oral glucocorticoid treatment as causal factor for the bone parameters and structure alteration. It was also noted that oral glucocorticoid therapy increased the risk of fractures particularly those of the vertebral body [30]. The present study is the first to evaluate and compare the effects of pearl millet and alendronate in lumbar vertebrae of rats fed on cafeteria diet associated with glucocorticoid. The groups receiving CAF and MPN pharmacological doses (9 mg/kg/b.w) show bone loss, and decreased lumbar trabecular bone (figure 3, B1 and C1). We noted equally that the number of osteocytes lacunae significantly decreased in MPN group compared to the control group. Our results also showed that bone marrow was decreased to favor the increase of fat microvacuoles in CAF and CAF-MPN groups. Banu et al reported similar results [31]. Deterioration of the trabecular architecture has been implicated in decreased bone strength and increased fracture incidence in humans [32].

Bisphosphonates such alendronate prevent bone loss at both lumbar spine and hip, and can further reduce the risk of vertebral fractures [33]. It has now been widely reported that alendronate can contribute to a higher level of resistance to induced-osteoporosis factors [34]. In other studies, long-term treatment of human and nonhuman primates with alendronate has shown increase wall thickness, reduce bone resorption and prevent glucocorticoid-induced osteoporosis [35, 36].

In our research we noted that histological evaluation in CAF-MPN-ALN and CAF-MPN-AEPG groups showed a greater improvement of lumbar trabecular bone and osteocyte lacunae compared to CAF and CAF+MPN groups.

In this regard, we noted that the AEPG increased lumbar trabecular bone and attenuated bone erosion, which shows a beneficial effect on bone fragility induced by methylprednisolone treatment.

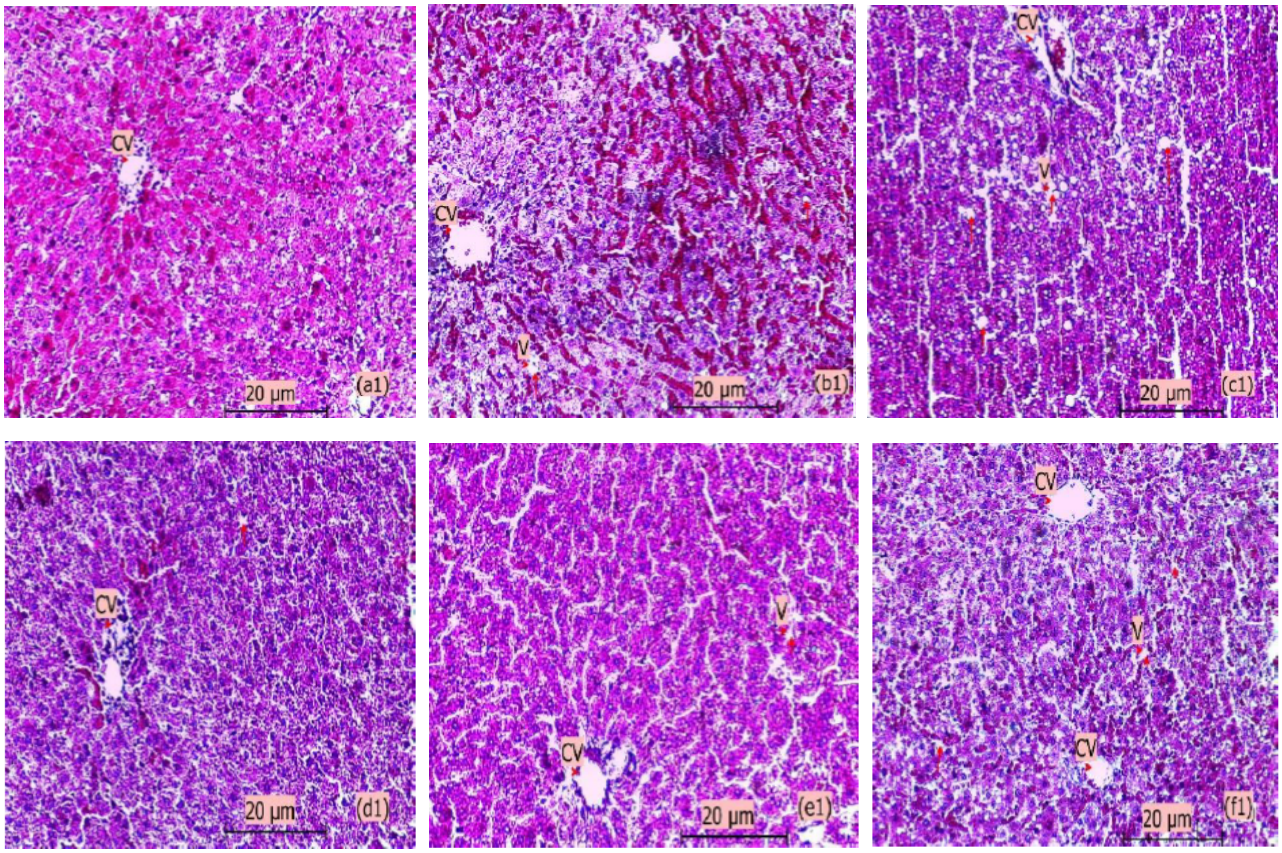

Figure 2. Effects of AEPG and ALN on liver of rats feed cafeteria diet and methylprednisolone. Manson's trichrome staining (200 × magnifications). Scale bar 20 µm: (a1) control group, (b1) CAF group, (c1) CAF+GC group, (d1) CAF+AEPG group, (e1) CAF+MPN+AEPG group, (f1) CAF+MPN+ALN group. The red arrow heads indicate fat vacuole. CV: central vein, V: vacuole.

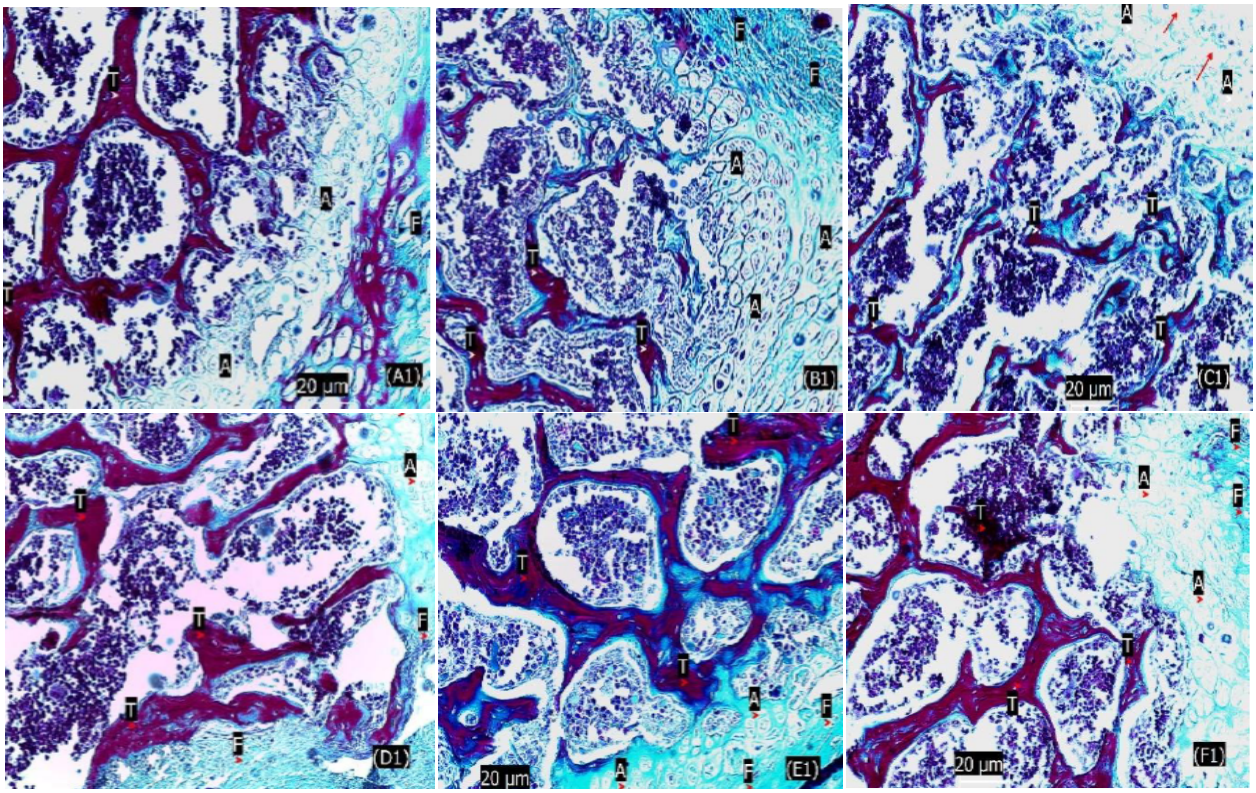

Figure 3: Effects of AEPG and ALN on lumbar vertebrae of rats feed cafeteria diet and methylprednisolone. Manson's trichrome staining (200 × magnifications). Scale bar 20 µm: (A1) control group, (B1) CAF group, (C1) CAF+MPN group, (D1) CAF+AEPG group, (E1) CAF+MPN+AEPG group, (F1) CAF+MPN+ALN group. The red arrow heads indicate collagen fiber. T: trabecular bone, F: collagen fiber, A: nascent bone

3.4. *Scanning electron microscopy evaluation*

Scanning electronic microscopy appreciates the bone matrix quality [37]. It shows that osteocyts lacunae orientations and lamellae structures are less ordered and aligned in rats fed on hyperlipidic diet [38].

In this study, we examined the samples by scanning electron microscopy. We found that CAF diet and CAF+MPN diminished the quality of bone structure, and increased bone alterations compared to the control group. At high magnification microfissures were observed at the surface of cortical bone in CAF+MPN group compared to control group. We noted that AEPG administration showed an increased structure area of trabecular bone and more volume thickness compared with the CAF+MPN group. This improvement was also observed in trabecular bone in alendronate treatment group compared to the CAF and CAF+MPN groups respectively (figure 4, F). It seems that AEPG prevents MPN-induced alterations in bone morphology. Our results reported that the comparison between AEPG and ALN showed no significant difference in bone improvement. We noted that methylprednisolone accelerate the bone marrow alteration in rat fed cafeteria diet. This phenomenon seems improve after AEPG and ALN treatment.

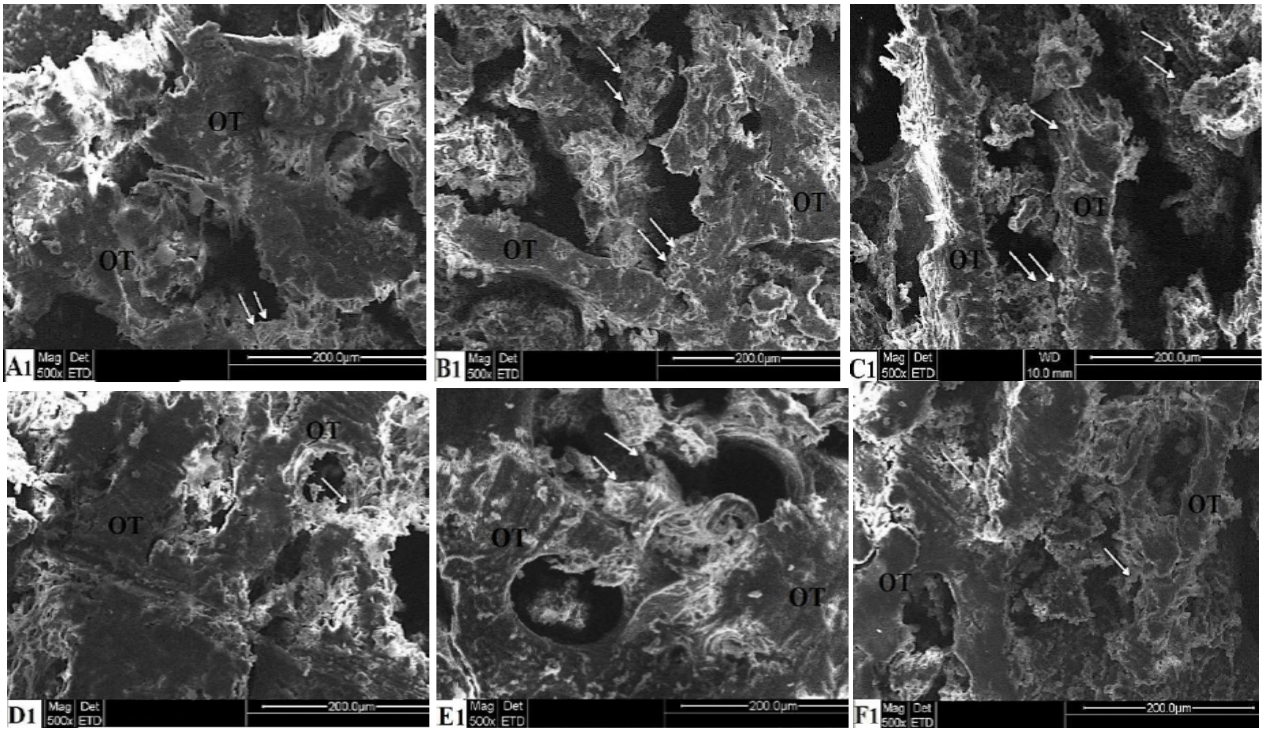

Figure 4: Effects of AEPG and ALN on lumbar vertebrae of rat feed cafeteria diet and methylprednisolone. (A) Control group; (B) CAF group; (C) CAF+MPN group; (D) CD+AEPG group; (E) CAF+MPN+AEPG group; CAF+MPN+ALN group. Scanning electronic microscopy images (500× magnifications); OT: trabecular. White arrows denote the remodeling areas defect.

Conclusion

According to the findings of this study, it is evident that *Pennisetum glaucum* has a beneficial effect on lumbar vertebrae osteoporosis induced by glucocorticoid therapy.

By this work, we have contributed to bring scientific proves of antiporotical activity of AEPG seed which is empirically used in traditional medicine. Furthermore, AEPG shows a similar effect to a bisphosphonate widely used to prevent and slow the progress of bone loss and fractures: the Alendronate and AEPG could be used as a new therapeutic pathway, inexpensive, natural alternative without adverse effects, in the bone loss and osteoporosis treatment new strategy and prevention.

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Conflict of Interest statement-The authors report no conflict interest.

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