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Piper sarmentosum Improves Bone Structure and Biomechanical Strength of Rats Given Excess Glucocorticoid

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Authors' contributions

This work was carried out in collaboration between all authors. SIN edited the manuscript MRES designed the study, wrote the first draft of the manuscript and answered the reviewer's comments, MASF and MA performed the research work the statistical analysis, wrote the protocol, and contributed the first draft of the manuscript. AF and HSF managed the literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To determine the effect of *Piper sarmentosum* (*Ps*) leaf extract on biomechanical strength and trabecular structure of the bones of glucocorticoid-induced osteoporotic rats.
Study Design: Administration of crude extract to rats with excessive glucocorticoids.
Place and Duration of Study: Department of Anatomy and Pharmacology, National University of Malaysia, between September 2010 and December 2011.
Methodology: Three-month old male *Sprague-Dawley* rats were adrenalectomized to remove the main source of circulating glucocorticoids. The animals were replaced with dexamethasone 120 µg/kg body weight/day. Treatment with *P. sarmentosum* 125 mg/kg body weight and glycirrhizic acid (GCA) 120 mg/kg body weight were given simultaneously for 2 months. After been sacrificed, a three-point bending configuration test for assessing the biomechanical properties of the right femoral bones was done using an Instron Universal testing machine equipped with Instron Bluehill software. The left undecalcified femoral bones were embedded in resin, sectioned and stained with Von

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Kossa for structural histomorphometric measurements.
Results: *P. sarmentosum* extract had significantly increased the intrinsic parameter (flexure modulus) and extrinsic parameter (energy at break) of the biomechanical properties of the bone. It had also significantly improved the trabecular structure by increasing the BV/TV, Tb.Th, Tb.N and by reducing the Tb.Sp based on histomorphometric analysis.
Conclusion: *P. sarmentosum* extract was able to protect bone biomechanical strength in glucocorticoid-induced osteoporotic bone, as confirmed by the structural histomorphometric finding. Therefore, *Ps* extract has the potential to be used as an agent to protect the bone strength and structure against osteoporosis due to chronic glucocorticoid treatment. These results however, need further study for better justification.

Keywords: Piper sarmentosum; biomechanical strength; bone histomorphometry; glucocorticoid; osteoporosis; dexamethasone.

1. INTRODUCTION

Long term exogenous glucocorticoid therapy causes profound reduction of bone mineral density (BMD), bone quality, bone formation and bone mechanical properties which leads to fracture (Raisz, 2005). Changes are more pronounced at the appendicular skeleton compared to the spine. The effects of glucocorticoids depend on the dose and duration of treatment. In glucocorticoid-induced osteoporosis, fracture occurs at higher BMDs than in postmenopausal osteoporosis (Van Staa et al., 2003). Fifty per cent of the patients chronically treated with glucocorticoids will suffer fractures (Manolagas and Weinstein, 1999). These effects are partially reversible upon discontinuation of therapy (Pierrotti et al., 2008). The rate of fracture depends more on bone microarchitecture rather that it's mineral content (Van Staa et al., 2002). Bone loss in glucocorticoid-induced osteoporosis is most pronounced in trabecular bone and cortical shell of vertebral bodies which leads to vertebral compression fractures (Canalis et al., 2007). Glucocorticoids inhibit osteoblastogenesis and reduce the lifespan of osteoblasts and osteocytes. They are also potent suppressors of osteoblast function and these cause reduction in bone formation (Weinstein et al., 1998). On the other hand glucocorticoids also increase osteoclastogenesis and prolong the life span of osteoclasts, which cause increased bone resorption leading to glucocorticoid-induced osteoporosis (Lems et al., 1998; Prummel et al., 1991). Study had shown that glucocorticoids antagonized bisphosphonate-induced osteoclast apoptosis (Weinstein et al., 2002). Moreover, glucocorticoids enhance the differentiation of macrophage/monocytes into mature osteoclasts. On the other hand, under in vitro conditions, glucocorticoids promote osteoclast generation and activation (Kaji et al., 1997). Glucocorticoids did not prolong osteoclast viability and did not decrease bone mass in TRAP-11β-HSD2 transgenic mice, hence provided the first in-vivo evidence that glucocorticoids act directly on osteoclasts leading to early bone loss (Jia et al., 2006).

Osteoporosis is characterized by reduced bone mass and deteriorated architecture and material property which leads to brittle bones that contribute to increase fracture risk (Kleerekoper et al., 1985). The bones can be made stronger by increasing the bone mass, distributing the bone mass effectively and by improving the material properties of bone tissue (Turner, 2002).

An ideal drug to treat osteoporosis would improve bone strength and decrease the bone brittleness. Most anti-osteoporotic drugs are either bone resorption inhibitors or stimulators of bone formation which will reduce the fracture risk. The current anti-osteoporotic drugs are mainly bone resorption inhibitors that acts to stabilize bone mass. These drugs reduce bone turnover causing an increase in bone mineral density and prevent further risk of fracture. This will increase the mean tissue age and bone mineralization (Meunier and Boivin, 1997). Increased mineralization will increase bone stiffness and decrease the ultimate displacement (Currey, 1969, 1990). Consequently it will improve the structural rigidity but at the same time will result in more brittle bone tissue (Currey, 1990). Anabolic agents which are able to increase bone remodeling in favor of bone formation rather than bone resorption are required to restore the density of osteoporotic bone.

Bone strength is determined by bone size, shape and material properties (Van der Muelen et al., 2001). Bone fragility is the susceptibility of the bone to fracture which depends on several biomechanical properties. Bone fragility is determined by three components: strength, brittleness and work to failure. Stiffness does not directly measure fragility but is used to assess mechanical integrity. Bone fragility can be reduced by increasing bone mass, distributing bone mass effectively which are the extrinsic properties; and by improving material properties of bone tissues which are the intrinsic biomechanical properties (Turner and Burr, 1993). Intrinsic biomechanical properties include ultimate stress and strain, Young's modulus and modulus of toughness (Turner et al., 1999). Bone quality is characterized by measurements of intrinsic biomechanical properties. Relevant modification of bone quality will lead to change in bone biomechanical properties which ultimately lead to fracture (Carter and Beuapré, 1990). An effective treatment of bone fragility should improve the extrinsic biomechanical properties and at the same time does not impair the intrinsic properties. An ideal drug to cure bone fragility would improve strength and decrease brittleness.

11β-hydroxysteroid dehydrogenase (11β-HSD) is known as an important pre-receptor signaling pathway in corticosteroid hormone action by catalyzing the interconversion of hormonally active cortisol in man (corticosterone in rodent) to inactive cortisone in man (dehydrocorticosterone in rodent) (White et al., 1997). Two isoenzymes of 11β-HSD, 11β-HSD1 and 11β-HSD2, have been shown to regulate glucocorticoid and mineralocorticoid hormone action (Stewart and Krozowski, 1999). 11β-HSD type 1 is a low-affinity bidirectional nicotinamide adenine dinucleotide phosphate (NADP[H])-dependant enzyme that can interconvert active cortisol to inactive cortisone; acts predominantly as a reductase in-vivo by converting inactive cortisone to active cortisol. In contrast, 11β-HSD type 2 is a high-affinity unidirectional nicotinamide adenine dinucleotide (NAD)-dependant dehydrogenase inactivating cortisol to cortisone; reductase activity for physiological glucocorticoids has not been seen for 11β-HSD2. Eijken et al., 2005 demonstrated that continuous treatment with dexamethasone induces differentiation of human osteoblast cell line (SV-HFO) cells during 21 days of culture. This showed that there is an inverse relationship between 11β-HSD1 activity and osteoblast differentiation. 11β-HSD1 is responsible for the local generation of glucocorticoids in bone which is expressed in human osteoblasts and osteoclasts (Cooper et al., 2000). It interconverts inactive cortisone to active cortisol in humans and inactive 11-dehydrocorticosterone to active corticosterone in rodents. Reductase activity in osteoblasts provides a potent mechanism for local generation of active glucocorticoids while the dehydrogenase activity attenuates local availability of active glucocorticoids. Increasing 11β-HSD1 dehydrogenase activity leads to significant decrease in bone resorption markers (Elvy Suhana et al., 2011).

11β-HSD1 is inhibited by liquorice and its derivatives, carbenoxolone and glycyrrhizic acid (Stewart et al., 1990). A study demonstrated that transgenic mice overexpressing 11β-HSD1 developed central obesity and metabolic syndrome (Masuzaki et al., 2001). Thus, 11β-HSD1 inhibitors may have beneficial effects in the treatment of glucocorticoid-induced osteoporotic rats.

Piper sarmentosum (*Ps*), locally known as "daun kadok" is a terrestrial herb, usually used to flavor local cuisine in South East Asia. The extract of the different parts of *Ps* is known to have potential benefits. *Ps* exhibited high antioxidative activities by measuring ferric reducing antioxidant potential assay (FRAP), DDPH free radical scavenging assay (DDPH) and β-carotene bleaching assay (Yusuf Sumazian et al., 2010). *Ps* leaf extract contains naringenin, a natural antioxidant compound with superoxide scavenging activity (Vimala et al., 2003). It posses an antioxidant, antiplasmodial, antituberculosis, anti-inflammatory, anticarcinogenic and hypoglycemic properties (Shahrul Hisham et al., 2009; Peungvicha et al., 1998). Furthermore, aqueous extract of *Ps* revealed anti-nociceptive and anti-inflammatory activities in vivo (Zakaria et al., 2010). Aida Azlina et al., 2009 have showed that *P. sarmentosum* water extract at 125mg/kg/day has the ability to inhibit 11β-HSD1 activity in the liver and adipose tissue of ovariectomized rats. Our recent study found that *Ps* extract increased the dehydrogenase activity of 11β-HSD1 and reduced the expression of 11β-HSD1 in the bones of glucocorticoid treated adrenalectomized rats (Elvy Suhana et al., 2012). These effects had caused reduction in the bone resorption marker (pyridinoline) and plasma corticosterone level in glucocorticoid treated rats (Elvy Suhana et al., 2011). A study on biomechanical evaluation of fracture healing in ovariectomized rats treated with *Ps* improved the strength and stiffness of bone by restoring its biomechanical properties (Estai et al., 2012). The histological analysis of the ovariectomized rats fed with *Ps* extract revealed that the fracture callus score was higher compared to the ovariectomized control group. Thus, indicate that *Ps* might have enhanced the healing of osteoporotic fracture (Estai et al., 2011).

Hence, the aim of this study was to evaluate the protective effects of *Ps* on bone biomechanical strength and trabecular structure through histomorphometry analysis. The results of this study may have an important impact in improving the bone structure and strength of patients on long term glucocorticoid treatment.

2. MATERIALS AND METHODS

2.1 Animals and Treatment

All procedures were carried out in accordance with the institutional guidelines for animal research of the Universiti Kebangsaan Malaysia UKM Research and Animal Ethics Committee (UKMAEC) (No: PP/ANT/2010/ELVY/14-JULY/313-JULY-2012-MAY-2012).

Thirty two, 3 months-old male *Sprague-Dawley* rats weighing 250-300 grams were obtained from the National University of Malaysia Animal Breeding Centre. The animals were divided into groups of eight rats and given the following treatment: Sham, sham-operated control given intramuscular vehicle olive oil 0.05 ml/kg and normal saline 1 ml/kg by oral gavage; AC, adrenalectomized control group given intramuscular dexamethasone 120 µg/kg/day and vehicle normal saline 1 ml/kg by oral gavage; AG, adrenalectomized rats given intramuscular dexamethasone 120 µg/kg/day and glycirrhizic acid (GCA) and AK, adrenalectomized rats given intramuscular dexamethasone 120 µg/kg/day and *Ps* extract 125 mg/kg/day by oral

gavage. Adrenalectomy was done two days after receiving the animals. The animals were first anaesthetized with Ketapex 5mg/100g body weight and Xylazil 1mg/100g body weight (Troy Laboratories, Australia). Dorsal midline and bilateral flank muscle incisions were then made and the adrenal glands were identified and removed. The incisions were sutured and Poviderm cream (Hoe Pharmaceuticals, Malaysia) was applied to the wound daily to prevent infection and aid wound healing. The rats were also given intramuscular injection of Baytril 5% (Bayer Health Care, Thailand) as prophylaxis for 5 days to prevent infection. The sham-operated rats underwent a similar procedure except that the adrenal glands were left in-situ. The treatment period was started 2 weeks after adrenalectomy. Dexamethasone (Sigma, USA) was dissolved in olive oil (Bertolli, Italy) and administered intramuscularly (120 µg/kg/day) for 6 days a week (Ima-Nirwana et al., 1998; Al-Wahaibi et al., 2007). The crude *Ps* extract was provided by the Forest Research Institute of Malaysia (FRIM). *Piper sarmentosum* and GCA (Sigma, USA) were diluted in normal saline and administered by oral gavage at the dose of 125 mg kg-1 and 120 mg kg-1 respectively for two months (Aida Azlina et al., 2009; Elvy Suhana et al., 2011; Estai et al., 2012). The sham-operated rats were administered equivalent volumes of vehicle olive oil intramuscularly and vehicle normal saline by oral gavage. The dexamethasone control (AC) rats were also administered vehicle normal saline by oral gavage. The administration of *Ps*, GCA and dexamethasone were started simultaneously two weeks days after the adrenalectomy. The treatment was given for two months. The animals were placed in clean cages under natural sunlight and darkness at night. They were given rat pellets (Gold Coin, Malaysia) ad libitum. The sham-operated animals were given tap water, while the adrenalectomized animals were given normal saline to drink ad libitium. This is to replace the salt loss due to mineralocorticoid deficiency post-adrenalectomy. At the end of two months of treatment, the rats were sacrificed humanely and the right femoral bones were taken for biomechanical test and the left for bone histomorphometry.

2.2 Bone Biomechanical Test

After the rats were sacrificed the right femur were cleaned from the surrounding tissues and wrapped in gauze dipped in phosphate-buffered saline. The bone has to be kept moist during the procedure. The biomechanical properties of the femoral bones were assessed using an Instron Universal testing Machine (model 5560, Instron, Canton, MA, USA) equipped with the Instron Bluehill software. The three-point bending configuration was used (Haffa et al., 2000), in which the bones were placed on two lower supports that are 10 mm apart. The force as applied at mid-diaphysis on the anterior surface such that the anterior surface as in compression and the posterior surface in tension until it fracture. The load, displacement stress and strain parameters were recorded by the software. Graphs of load against displacement and stress against strain were plotted. The slope-value of the stress-strain curve represented the elastic modulus of the femur. The main parameters of the bone mechanical test can be divided into extrinsic and intrinsic parameters: The extrinsic parameters (load, displacement and stiffness) measure the properties of whole bone and the intrinsic parameters (stress, strain and modulus of elasticity) measure the material of the bone (Turner, 2002).

2.3 Bone Histomorphometry

For structural histomorphometric measurements, which included trabecular bone volume (BV/TV) and trabecular thickness (Tb.Th), undecalcified bone samples were embedded in the mixture of Osteo Bed Resin Solution A (Polysciences Inc., PA, Germany) with Benzoyl

Peroxide Plasticized (Catalyst) (Polysciences Inc., PA, Germany) in the ratio of 100 ml of Osteo Bed Resin Solution A: 1.4g of Benzoyl Peroxide Plasticized (Catalyst). The samples were sectioned at the thickness of 9 µm using a microtome (Leica RM2155, Nussloch, Germany) and stained with the Von Kossa method. Structural parameters were analyzed by an image analyzer (Leica DMRXA2, Wetzlar, Germany) using the VideoTest-Master software (VT, St. Petersburg, Russia). Structural histomorphometric measurements were performed randomly at the metaphyseal region, which was located 3 - 7 mm from the lowest point of the growth plate and 1 mm from the lateral cortex, excluding the endocortical region. The selected area is known as the secondary spongiosa area, which is rich in trabecular bone. All parameters were measured according to the American Society of Bone Mineral Research Histomorphometry Nomenclature Committee (Parfitt et al., 1987).

2.4 Statistical Analysis

Data were tested for normality using the Shapiro-Wilk test. Since the groups were found to be normally distributed, the data was analyzed by parametric statistics, i.e. the ANOVA test followed by pos-hoc Tukey test for comparison between treatment groups. Statistical software used was the Statistical Package for Social Science (SPSS) version 19. Data were expressed as the mean ± standard error of the mean (SEM). The level of significant was set at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 *Piper sarmentosum* Enhances Bone Biomechanical Strength

Two months of dexamethasone treatment had significantly compromised both the intrinsic (flexure modulus, stress and strain) and extrinsic properties (load, stiffness and flexure extension). Rats supplemented with *Ps* (AK) had significantly improved both the intrinsic (flexure modulus) (Fig. 1) and extrinsic properties (energy) (Fig. 2) of the dexamethasone-treated adrenalectomized rats and comparable with GCA (AG) and sham groups. The *Ps* also had improved the stress (Fig. 3), strain (Fig. 4), load (Fig. 5) and flexure extension (Fig. 6) comparable with GCA and sham, but they were not statistically significant.

3.2 *Piper sarmentosum* Improves Bone Structural Histomorphometry

The Bone Volume/Tissue Volume (BV/TV) (Fig. 7, 11A & 11B), Trabecular Thickness (Tb.Th) (Fig. 8, 11A & 11B) and Trabecular Number (Tb.N) (Fig. 9, 11A & 11B) reduced significantly after 2 months in dexamethasone treated adrenalectomized rats with *Ps* (AK) and comparible with GCA (AG) and sham operated rats (Sham). Apart from that, there was also significant increase in Trabecular Separation (Tb.Sp) (Fig.10, 11A & 11B) in the dexamethasone treated adrenalectomized rats with *Ps* (AK). Supplementing the dexamethasone treated adrenalectomized rats with 125mg/kg/day *Ps* and 120mg/kg/day GCA had caused significant increase of BV/TV, Tb.Th and Tb.N. Apart from that, the Tb.Sp was also significantly reduced.

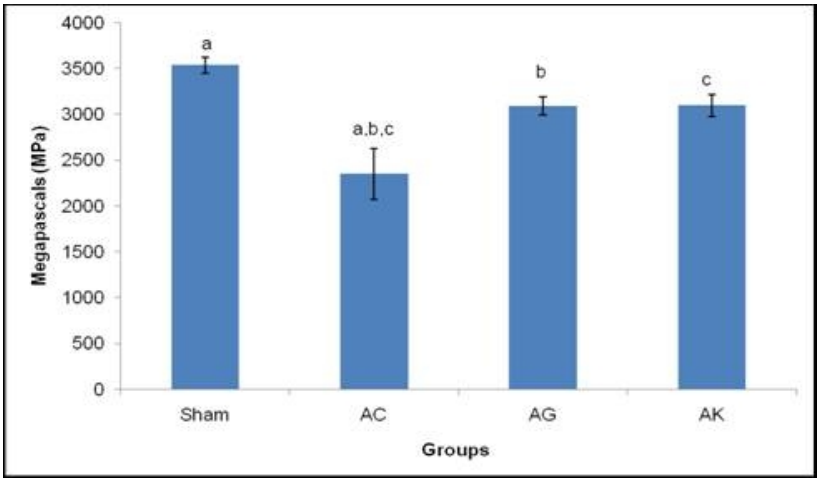


Fig. 1. The flexure modulus of femoral bones

(Sham) sham-operated group treated with normal saline, (AC) adrenalectomized (adrx) control group given intramuscular Dexamethasone (DEX) 120 µg/kg/day, (AG) adrx group given intramuscular DEX 120 µg/kg/day and treated with GCA 120 mg/kg/day, (AK) adrx group given intramuscular DEX 120 µg/kg/day and treated with Ps 125 mg/kg/day. n = 8. Data presented as mean ± SEM. Same alphabets indicate significant difference between groups at P < 0.05.

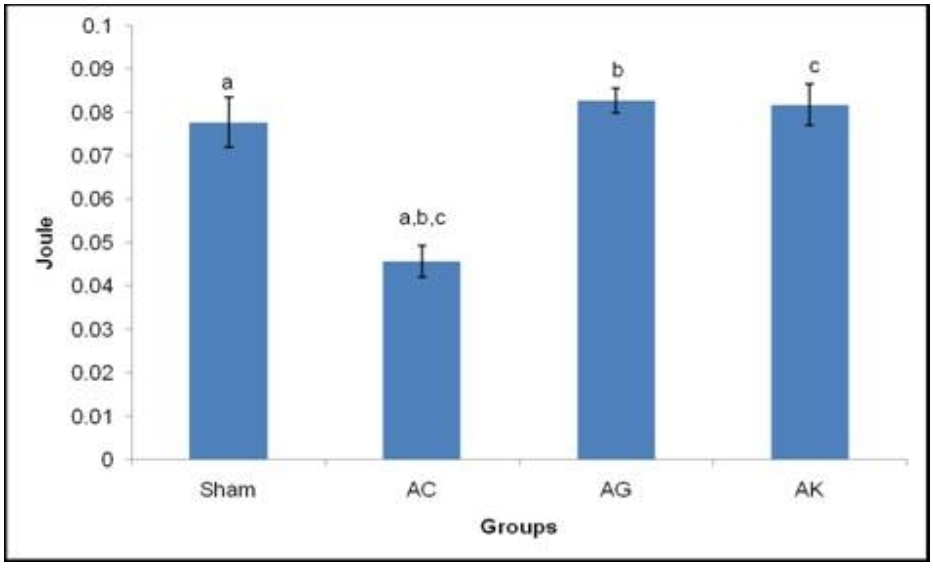


Fig. 2. The energy values of femoral bones

(Sham) sham-operated group treated with normal saline, (AC) adrx control group given intramuscular DEX 120 µg/kg/day, (AG) adrx group given intramuscular DEX 120 µg/kg/day and treated with GCA 120 mg/kg/day, (AK) adrx group given intramuscular DEX 120 µg/kg/day and treated with Ps 125 mg/kg/day. n = 8. Data presented as mean + SEM. Same alphabets indicate significant difference between groups at P <0.05.

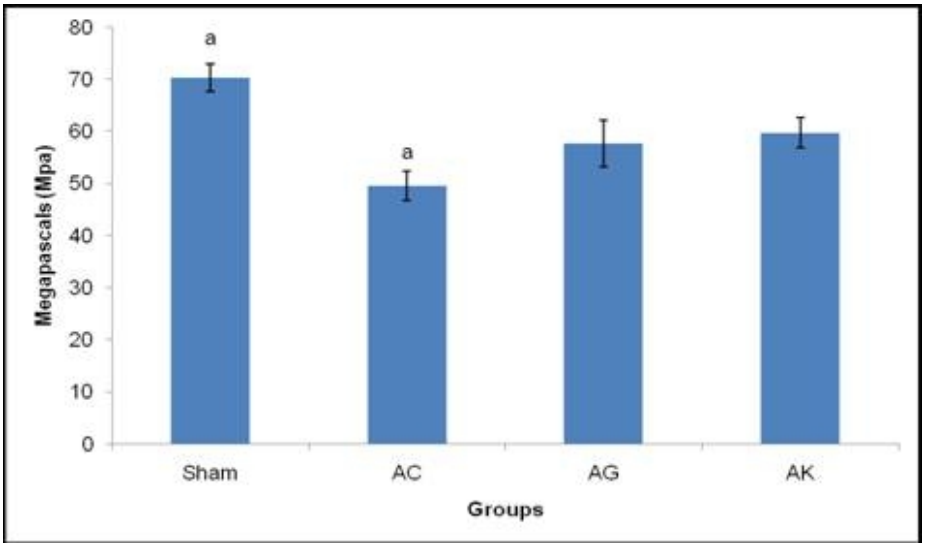


Fig. 3. The stress values of femoral bones

(Sham) sham-operated group treated with normal saline, (AC) adrx control group given intramuscular DEX 120 µg/kg/day, (AG) adrx group given intramuscular DEX 120 µg/kg/day and treated with GCA 120 mg/kg/day, (AK) adrx group given intramuscular DEX 120 µg/kg/day and treated with Ps 125 mg/kg/day. n = 8. Data presented as mean ± SEM. Same alphabets indicate significant difference between groups at P <0.05.

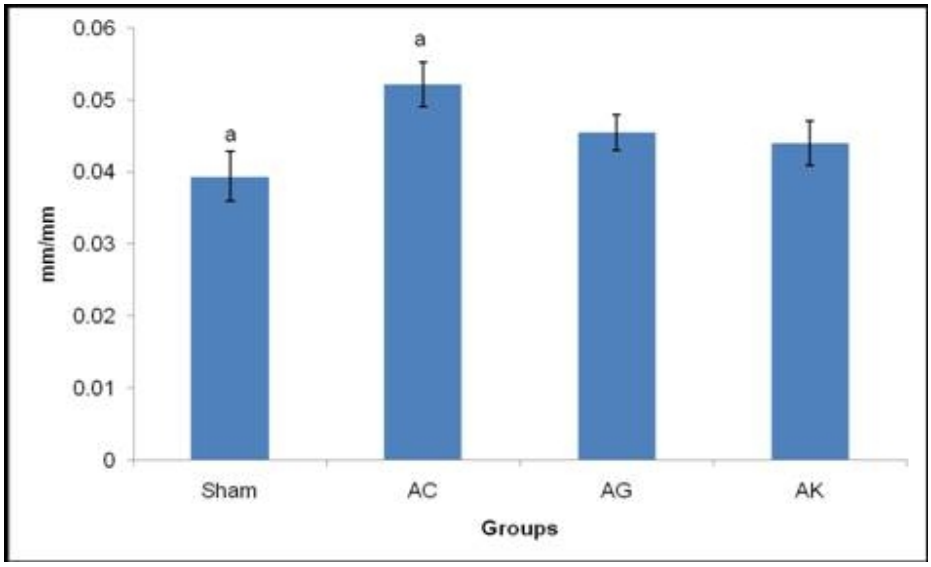


Fig. 4. The strain values of femoral bones.

(Sham) sham-operated group treated with normal saline, (AC) adrx control group given intramuscular DEX 120 µg/kg/day, (AG) adrx group given intramuscular DEX 120 µg/kg/day and treated with GCA 120 mg/kg/day, (AK) adrx group given intramuscular DEX 120 µg/kg/day and treated with Ps 125 mg/kg/day. n = 8. Data presented as mean ± SEM. Same alphabets indicate significant difference between groups at P <0.05.

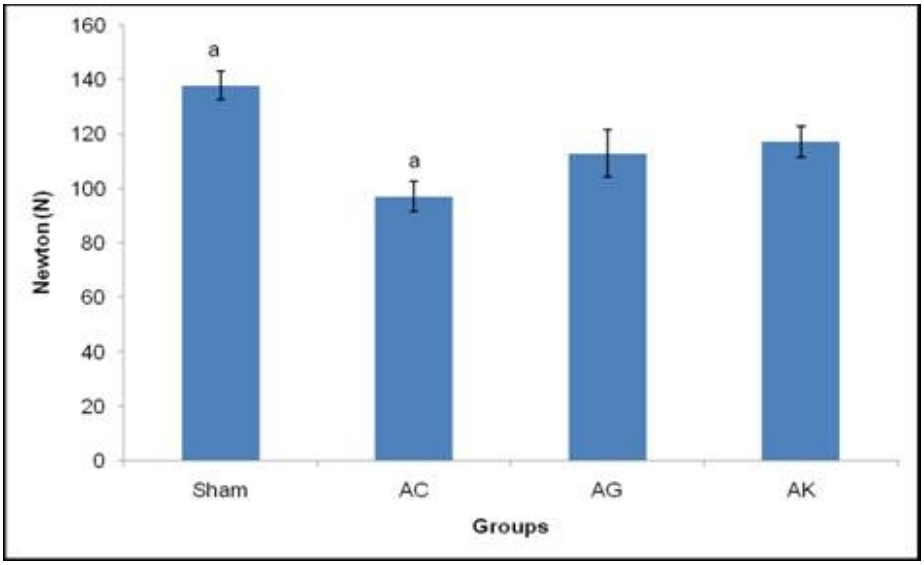


Fig. 5. The load values of femoral bones

(Sham) sham-operated group treated with normal saline, (AC) adrx control group given intramuscular DEX 120 µg/kg/day, (AG) adrx group given intramuscular DEX 120 µg/kg/day and treated with GCA 120 mg/kg/day, (AK) adrx group given intramuscular DEX 120 µg/kg/day and treated with Ps 125 mg/kg/day. n = 8. Data presented as mean ± SEM. Same alphabets indicate significant difference between groups at P <0.05.

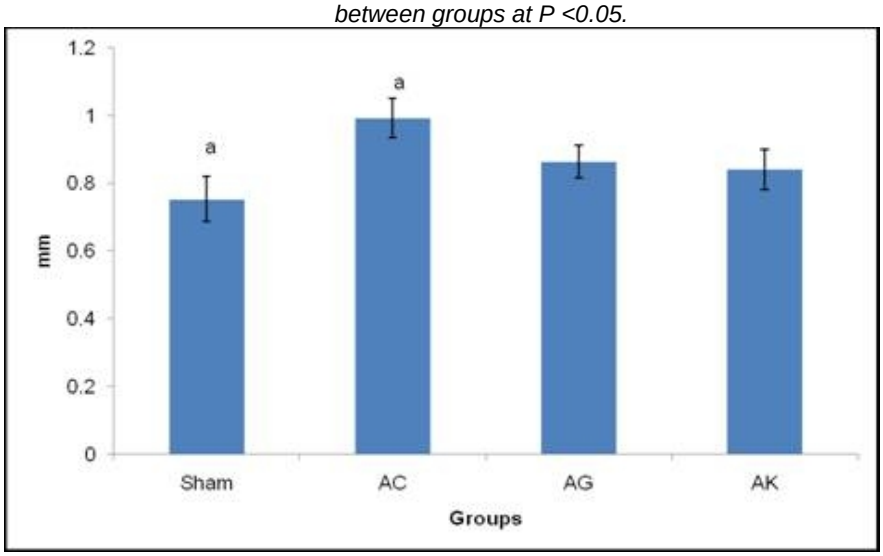


Fig. 6. The flexure extension of femoral bones

(Sham) sham-operated group treated with normal saline, (AC) adrx control group given intramuscular DEX 120 µg/kg/day, (AG) adrx group given intramuscular DEX 120 µg/kg/day and treated with GCA 120 mg/kg/day, (AK) adrx group given intramuscular DEX 120 µg/kg/day and treated with Ps 125 mg/kg/day. n = 8. Data presented as mean ± SEM. Same alphabets indicate significant difference between groups at P <0.05.

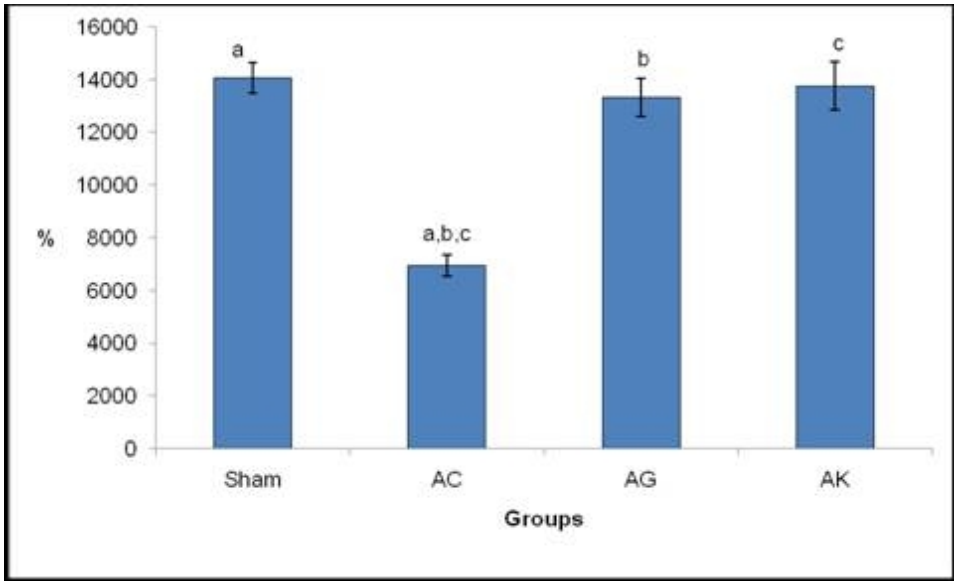


Fig. 7. The Bone Volume/ Tissue Volume (BV/TV) of femoral bones

(Sham) sham-operated group treated with normal saline, (AC) adrx control group given intramuscular DEX 120 µg/kg/day, (AG) adrx group given intramuscular DEX 120 µg/kg/day and treated with GCA 120 mg/kg/day, (AK) adrx group given intramuscular DEX 120 µg/kg/day and treated with Ps 125 mg/kg/day. n = 8. Data presented as mean ± SEM. Same alphabets indicate significant difference between groups at P <0.05.

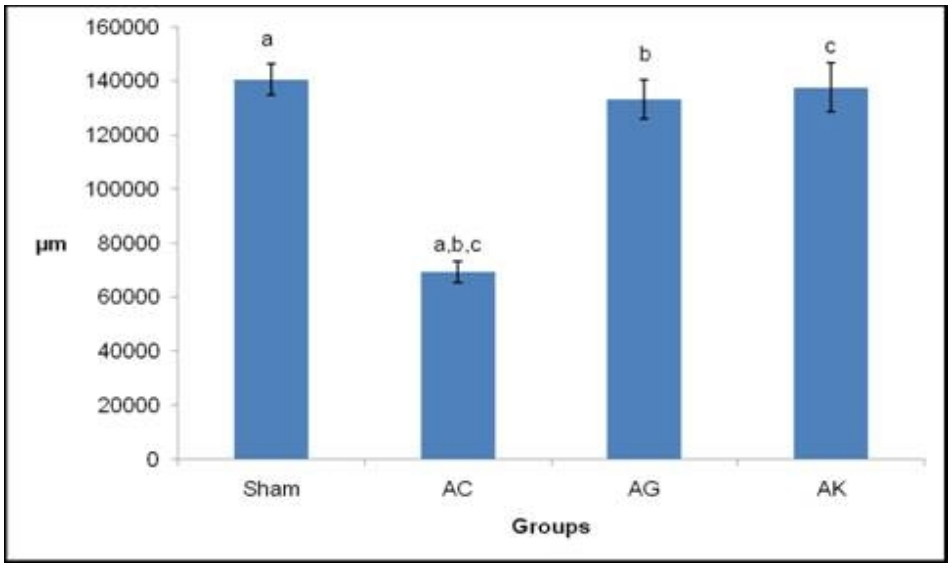


Fig. 8. The Trabecular Thickness (Tb.Th) of femoral bones.

(Sham) sham-operated group treated with normal saline, (AC) adrx control group given intramuscular DEX 120 µg/kg/day, (AG) adrx group given intramuscular DEX 120 µg/kg/day and treated with GCA 120 mg/kg/day, (AK) adrx group given intramuscular DEX 120 µg/kg/day and treated with Ps 125 mg/kg/day. n = 8. Data presented as mean ± SEM. Same alphabets indicate significant difference between groups at P <0.05.

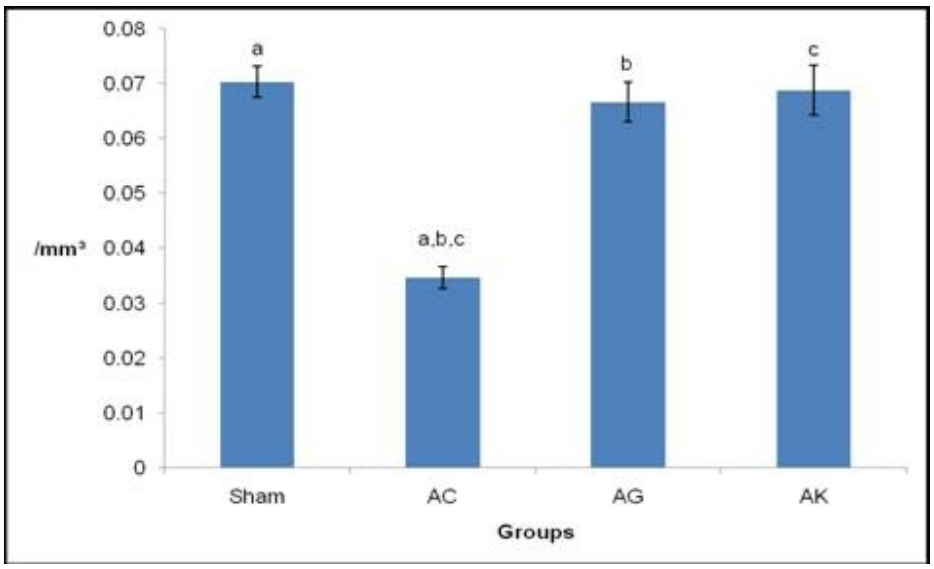


Fig. 9. The Trabecular Number (Tb.N) of femoral bones

(Sham) sham-operated group treated with normal saline, (AC) adrx control group given intramuscular DEX 120 µg/kg/day, (AG) adrx group given intramuscular DEX 120 µg/kg/day and treated with GCA 120 mg/kg/day, (AK) adrx group given intramuscular DEX 120 µg/kg/day and treated with Ps 125 mg/kg/day. n = 8. Data presented as mean ± SEM. Same alphabets indicate significant difference between groups at P <0.05.

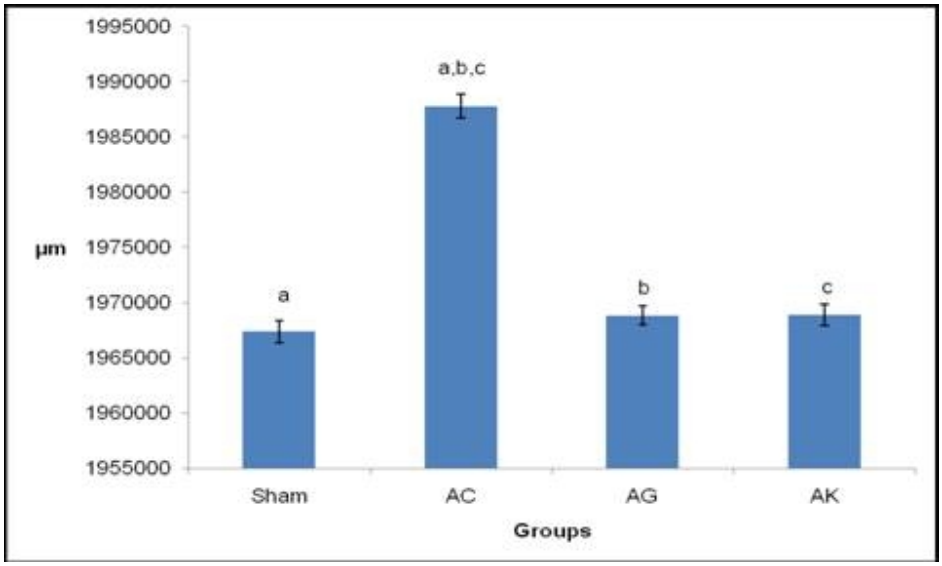
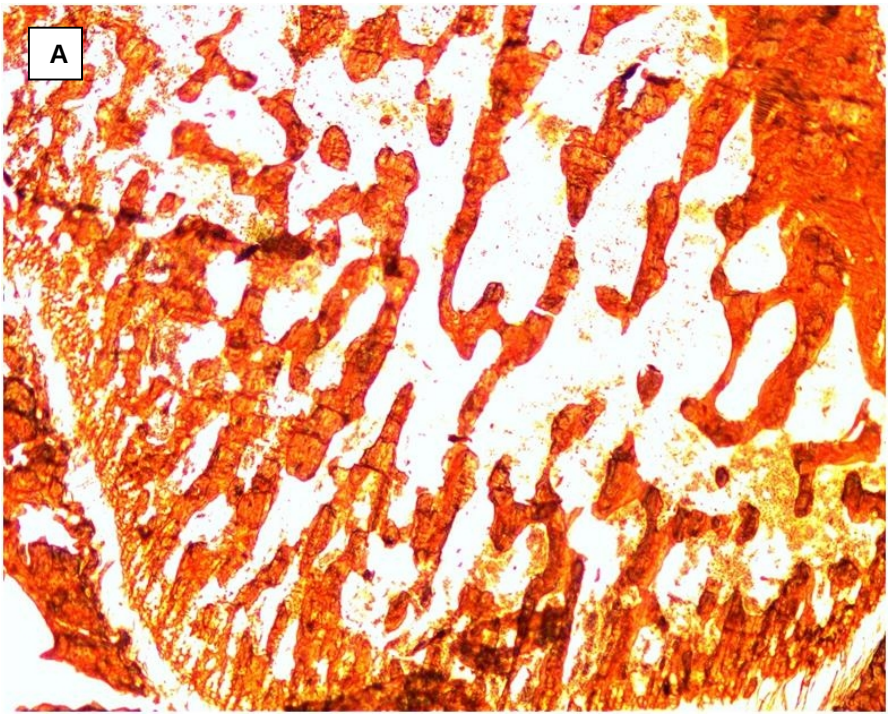


Fig. 10. The Trabecular Separation (Tb.Sp) of femoral bones

(Sham) sham-operated group treated with normal saline, (AC) adrx control group given intramuscular DEX 120 µg/kg/day, (AG) adrx group given intramuscular DEX 120 µg/kg/day and treated with GCA 120 mg/kg/day, (AK) adrx group given intramuscular DEX 120 µg/kg/day and treated with Ps 125 mg/kg/day. n = 8. Data presented as mean ± SEM. Same alphabets indicate significant difference between groups at P <0.05.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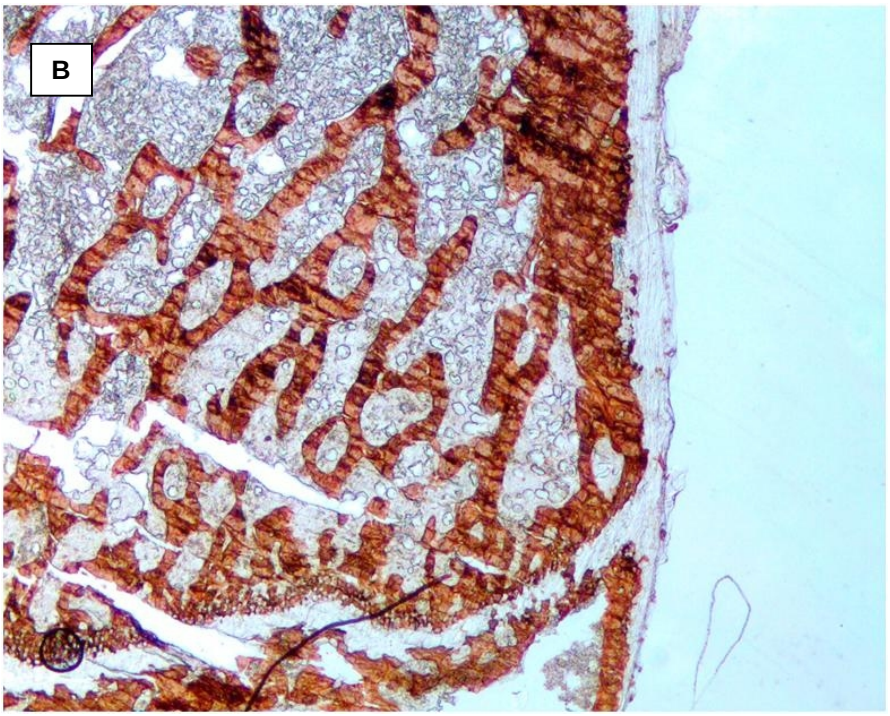
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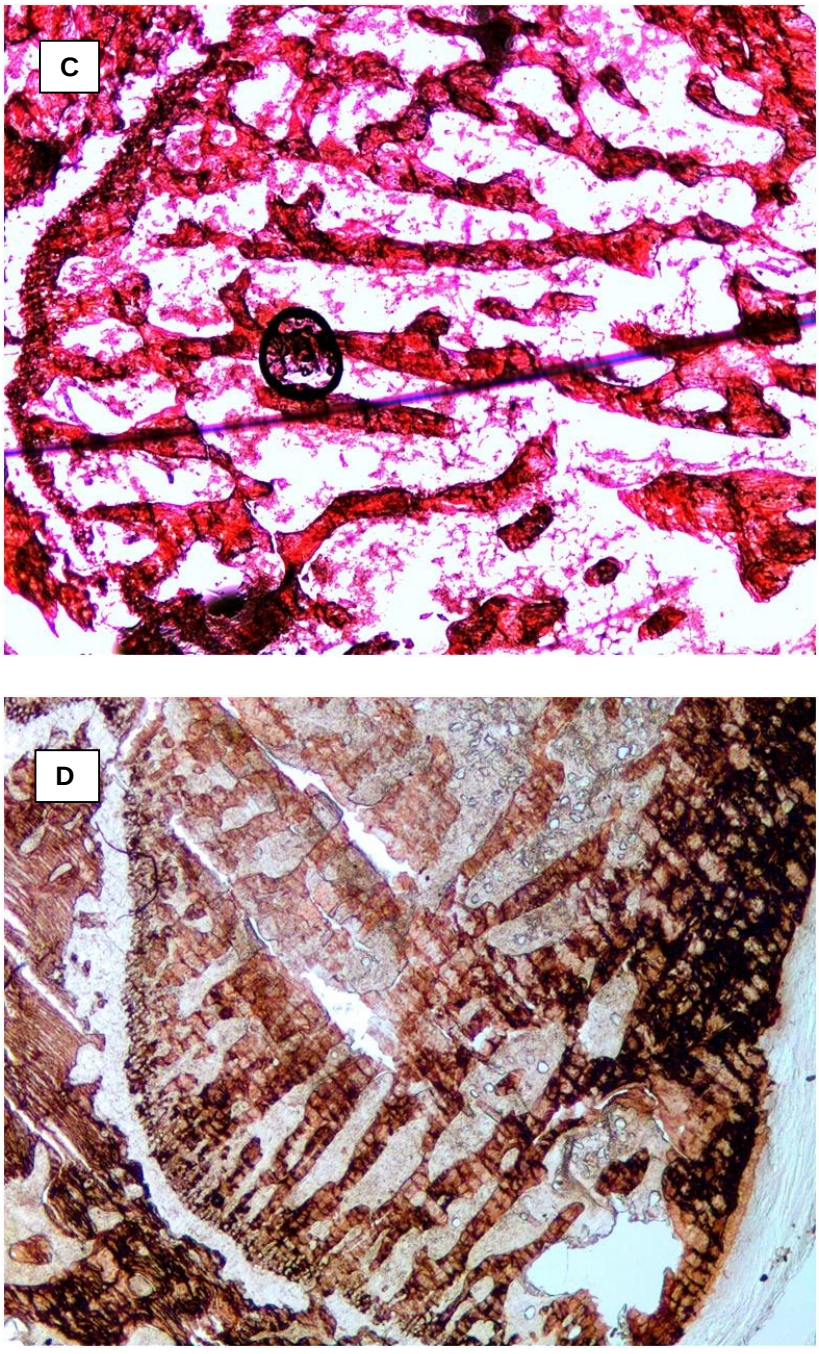
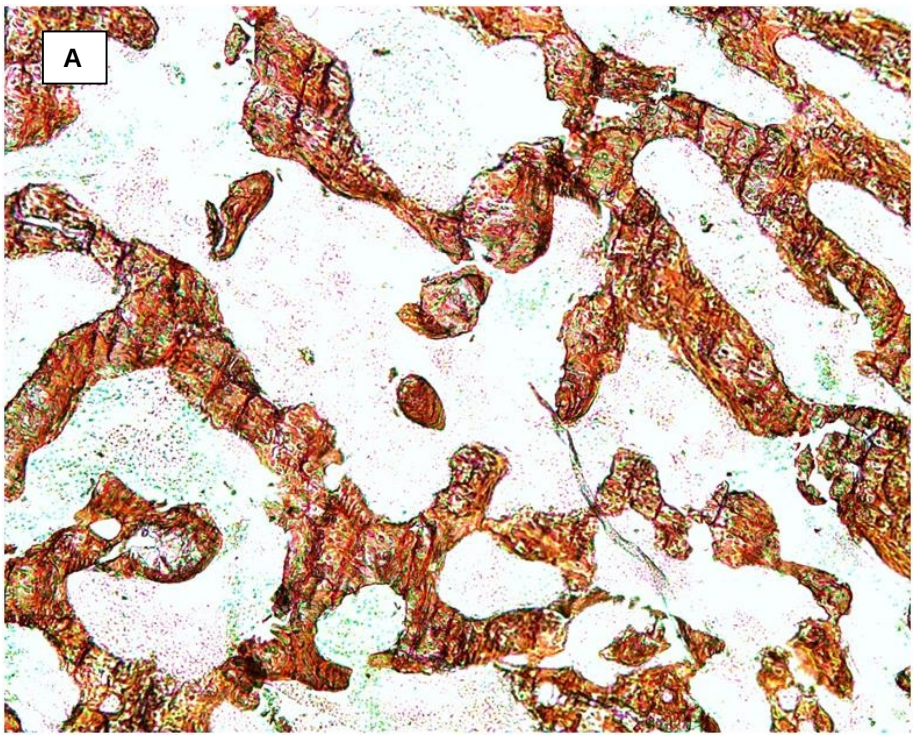
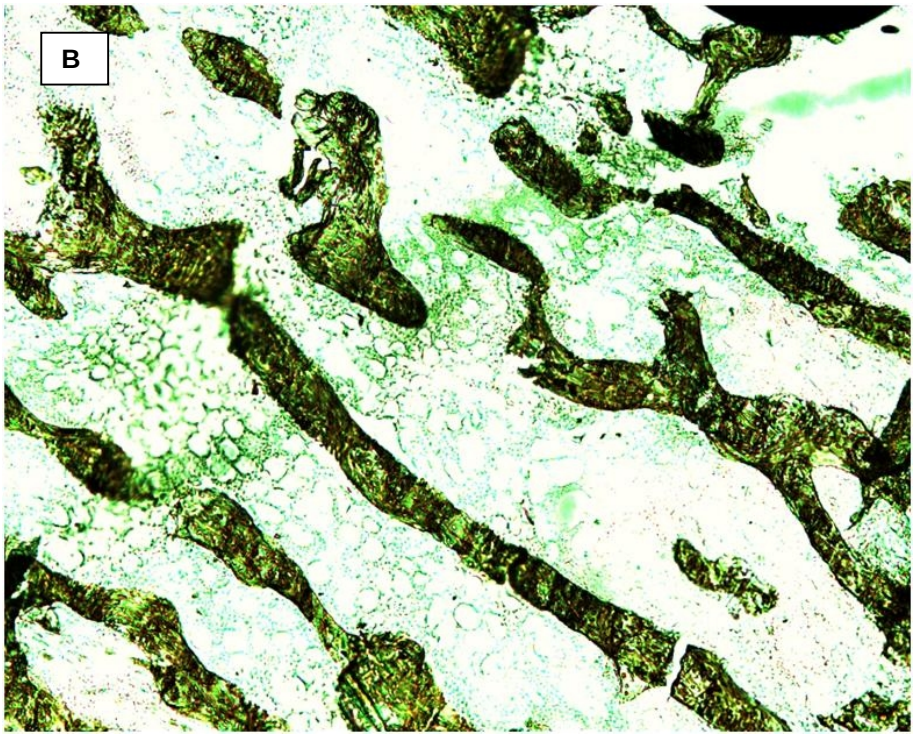


Fig. 11A. Photomicrographs showing trabecular bones with Von Kossa staining of undecalcified bone at 25x magnification

(A) sham-operated group treated with normal saline, (B) adrx control group given intramuscular DEX 120 µg/kg/day, (C) adrx group given intramuscular DEX 120 µg/kg/day and treated with GCA 120 mg/kg/day, (D) adrx group given intramuscular DEX 120 µg/kg/day and treated with Ps 125 mg/kg/day. Adrx-control group exhibit more separated and thinner trabecular bone.


A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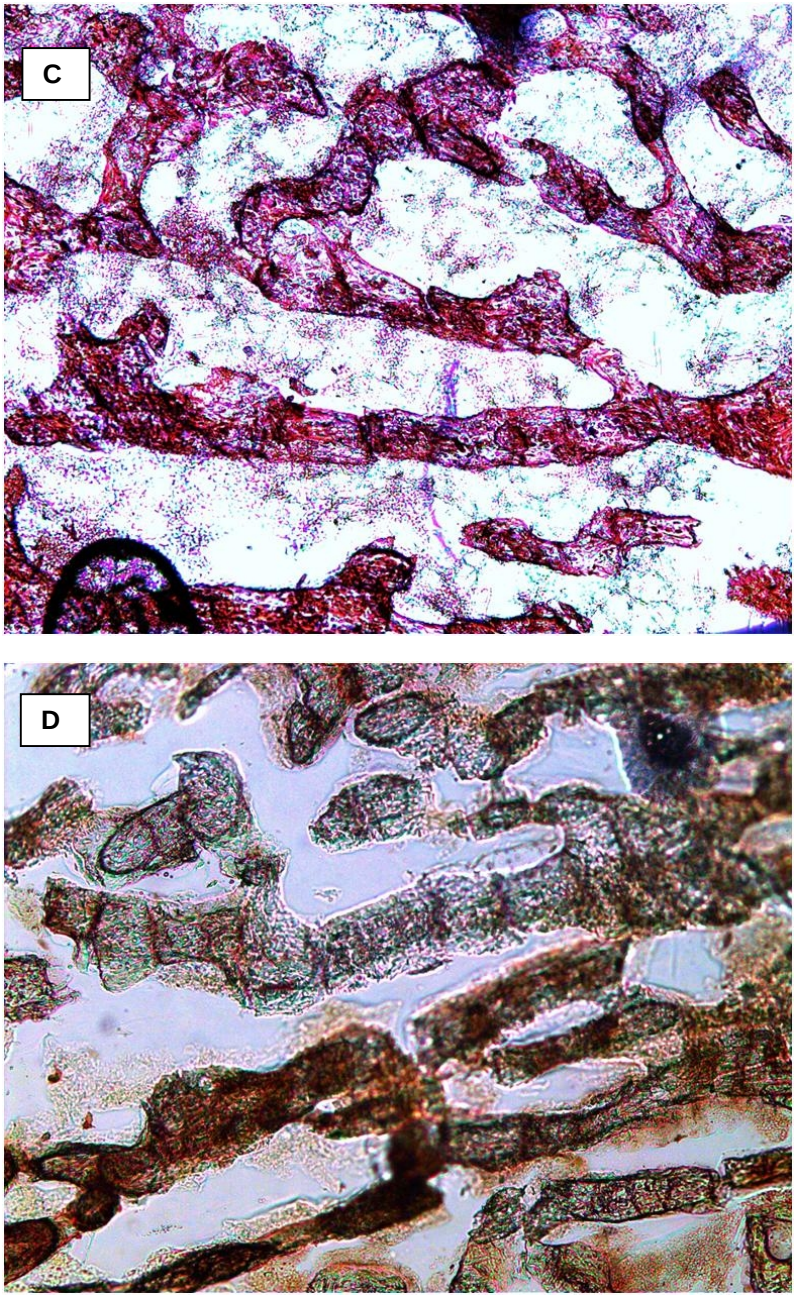

Fig. 11B. Photomicrographs showing trabecular bones with Von Kossa staining of undecalcified bone at 50x magnification

(A) sham-operated group treated with normal saline, (B) adrx control group given intramuscular DEX 120 µg/kg/day, (C) adrx group given intramuscular DEX 120 µg/kg/day and treated with GCA 120 mg/kg/day, (D) adrx group given intramuscular DEX 120 µg/kg/day and treated with Ps 125 mg/kg/day. Dexamethasone treated group exhibit more separated and thinner trabecular bone.

Our recent studies found that *Piper sarmentosum* has the potential to protect the bone against glucocorticoid-induced osteoporosis by regulating 11β-HSD1 expression and activity (Elvy Suhana et al., 2011, 2012). The 11β-HSD1 dehydrogenase activity in the bone was increased while the expression was reduced. Supplementation of *Ps* extract at the dose of 125mg/kg/day increased the dehydrogenase activity which will reduce the active glucocorticoid level in the bone (Cooper et al., 2000). Reduced active local glucocorticoid level had reduced the bone resorption as shown by decreased bone reseptor markers (Elvy Suhana et al., 2011). This study is done to confirm those findings by assessing its affect on trabecular bone structure and bone biomechanical strength. A similar dose of 125mg/kg/day of *Ps* extract and 120mg/kg/day of GCA were used.

Male rats were chosen as the influence of the reproductive hormones is less. Adrenalectomy was performed to the rats to remove the endogenous glucocorticoids which were under influence of circadian rhythm, physical and emotional stress. The endogenous glucocorticoids were replaced by predetermined doses of dexamethasone to establish a constant level of excess glucocorticoid in the body. No replacement of mineralocorticoids was given as it was shown in earlier studies that they have no influence on bone metabolism (Ima Nirwana and Fakhrurazi, 2002). However, the animals were given normal saline ad libitium to maintain normal sodium homeostasis. The dose and duration of dexamethasone treatment (120 µg/kg) were based on previous studies (Elvy Suhana et al., 2011; Ima Nirwana and Fakhrurazi, 2002; Ima Nirwana and Suhaniza, 2004). The dose of the GCA and *Ps* were also determined by previous studies (Aida Azlina et al., 2009; Al-Wahaibi et al., 2007). The successful induction of osteoporosis was demonstrated by histomorphometry and biomechanical strength test.

The findings of the biomechanical test showed that long term dexamethasone treatment had significantly weakened the bone compared to the sham operated rats. Long term dexamethasone treatment had compromised both the extrinsic (load, energy and flexure extension) and intrinsic parameters (flexure modulus, stress and strain). The reduction of biomechanical strength was associated with profound depression of bone formation as seen in bone histomorphometric analysis which showed significant reduction in trabecular volume, number and thickness less compact compared to the sham operated rats. The bone loss was associated with an increase in bone resorption, decrease in bone formation, impaired bone strength and histomorphometric changes of osteoporosis (Hofbauer et al., 1999). This could be explained that glucocorticoids treatment cause a time and dose dependent increase in 11β-HSD1 reductase activity (Cooper et al., 2002). Reduced activity of 11β-HSD1 dehydrogenase may imply increased activity of 11β-HSD1 reductase in bone, leading to increased local glucocorticoid production (Cooper et al., 2000). Increased glucocorticoid concentration in bone will lead to increased bone resorption and reduced bone formation (Weinstein, 2001). This will ultimately cause osteoporosis.

Based on the biomechanical strength test and histomorphometric analysis, supplementation of the dexamethasone-treated adrenalectomized rats with GCA which is a potent inhibitor of 11β-HSD1 had improved the bone strength. It also produced bone with better trabecular volume, thickness and number and was more compact compared to the nonsupplemented group. The suppression of bone loss may be due to GCA had increased the 11β-HSD1 dehydrogenase activity in the bone that could have reduced the active glucocorticoids levels in the bone as reported by previous studies (Elvy Suhana et al., 2011; Van Staa et al., 2002). The reduction of active glucocorticoids in the bone could have reduced the bone resorption and improved the bone mass, structure and strength. Eijken et al. (2005) in-vitro study showed that 11β-HSD1 reductase activity was blocked completely by 18β-

glycyrrhetinic acid. Cooper et al. (2000) also found that carbenoxolone, which is a liquorice derivative, had significantly reduced the production of bone formation markers. Glycyrrizic acid and carbenoxolone, as 11β-HSD inhibitors, prevent the augmentation of glucocorticoids levels within osteoclasts or their precursors.

Treatment of dexamethasone-treated adrenalectomized rats with *Ps* had given very similar results to those seen in the GCA group where it had improve the trabecular volume, number and thickness. Supplementation with *Ps* also had resulted in more compact bone which had enhanced the bone biomechanical properties. Therefore, we may conclude that *Ps* is effective in improving the bone strength of glucococorticoid induced osteoporosis in rats most probably by inhibiting the bone resorption. This is done through the stimulation of 11β-HSD1 dehydrogenase activity and expression in the bone (Elvy Suhana et al., 2012). In addition, an anti-osteoporotic effect of *Ps* aqueous extract had been observed in the adrenalectomized rats (Ima Nirwana et al., 2009). The beneficial effect of *Ps* on osteoporosis and fracture healing is most probably attributed to the anti-oxidative actions of the *Ps* flavonoids which may prevent oxidative stress. Thus there is a real potential for *Ps* to be used to prevent osteoporosis and fracture occurrence in patients on long term glucocorticoid therapy, subject to further animal and human studies.

4. CONCLUSION

Ps was effective in improving the bone structure and biomechanical properties in the rats with excess glucocorticoids, which may reduce the occurrence of fragility fractures. This suggest that *Ps* can be used as an agent to protect the bone against the action of glucocorticoid and this may benefit the patients who needs to be on long-term glucocorticoid treatment. However, the mechanisms involved need further exploration.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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