Journal of Basic and Clinical Physiology and Pharmacology

Decision Letter (JBCPP.2020.0140)

From: m.horowitz@mail.huji.ac.il

To: rr-retno-w@ff.unair.ac.id

CC: jbcpp.editorial@degruyter.com

Subject: JBCPP.2020.0140 - Decision Revise with Modifications

Body: 24-Jul-2020

Dear Dr. Widyowati:

Thank you again for submitting your manuscript ID JBCPP.2020.0140 entitled "The effect of deer antler from East Kalimantan to increase bone density that related to bone turnover" to Journal of Basic and Clinical Physiology and Pharmacology (JBCPP). Your manuscript has been reviewed and requires modifications prior to acceptance. The comments of the reviewer(s) are included at the bottom of this letter.

I invite you to respond to the reviewer(s)' comments and revise your manuscript. The revised paper needs to be submitted within 5 weeks from now.

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Once again, thank you for submitting your manuscript to JBCPP. I look forward to receiving your revision.

Kind regards
Dr. Michal Horowitz
Editor in Chief, Journal of Basic and Clinical Physiology and Pharmacology

Reviewer(s)' Comments to Author:

Reviewer: 1

Comments to the Author
Please see the attached two pdf files

Reviewer: 2

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Reviewer: 1

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The effect of deer antler from East Kalimantan to increase trabecular bone density and calcium levels serum on osteoporotic mice

Journal:	*Journal of Basic and Clinical Physiology and Pharmacology*
Manuscript ID	JBCPP.2020.0140.R1
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Complete List of Authors:	Widyowati, Retno; Universitas Airlangga Fakultas Farmasi, Pharmacognosy and phytochemistry Suciati, Suciati; Universitas Airlangga Fakultas Farmasi, Pharmacognosy and Phytochemistry Hariyadi, Dewi Melani; Universitas Airlangga Fakultas Farmasi, Pharmaceutics Chang, Hsin-I ; National Chiayi University, Biochemical Science and Technology Suryawan, IPG Ngurah; Dinas Peternakan dan Kesehatan Hewan, UPTD pembibitan dan inseminasi Buatan Tarigan, Nurliana; Dinas Peternakan dan Kesehatan Hewan, UPTD Pembibitan dan Inseminasi Buatan
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Acknowledgments

The authors are grateful for access to Animal Laboratory of the Faculty of Pharmacy, Universitas Airlangga and would like to express their sincere thanks to Research and Innovation Institute of Universitas Airlangga and the dean of Faculty of Pharmacy, Universitas Airlangga for supporting fund.

Research funding

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Author contributions

All authors have accepted responsibility for the entire content of this manuscript and approved its submission. RW designed the research concepts, SC designed methods, DW edited the article, HC designed discussion, NS and NT provided the deer antlers.

Competing interests

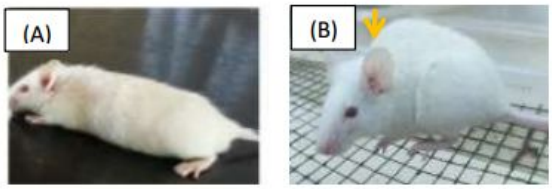
The authors declared that no conflict of interest in this article.

Informed consent

No informed consent was declared in this study.

Ethical approval

This study was approved by ethics commission of Faculty of Veterinary Medicine, Universitas Airlangga with No. 2.KE.176.09.2019



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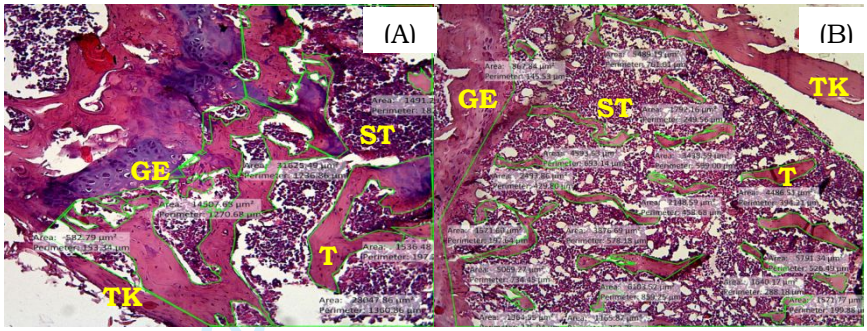

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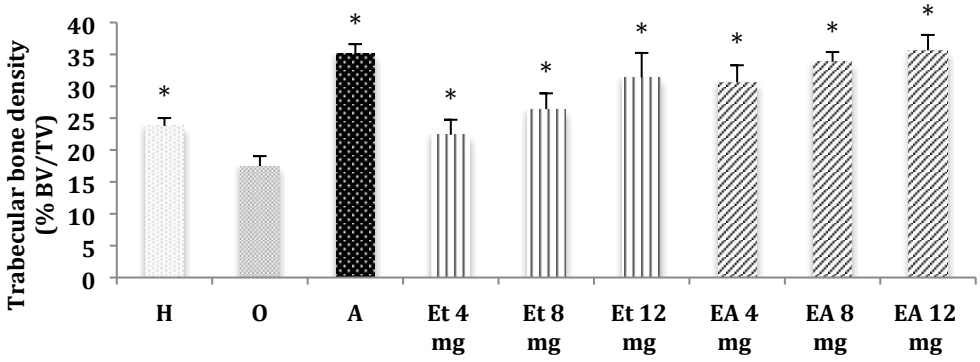

Figure 3. Trabecular bone density levels of several groups; healthy (H), osteoporotic (O), positive control (A, alendronate), 70% ethanol extracts (Et at 4 mg, 8 mg, and 12 mg), and aqueous extracts (EA at 4 mg, 8 mg, and 12 mg) after intervention, (*p<0.05).

Table 1. Calcium levels in serum of several groups after intervention (*p<0.05).

Groups	Calcium levels in serum (ppm)
Healthy	9.65±0.02*
Osteoporotic	9.15±0.04
Positive control	9.89±0.05*
70% ethanol extract:	
4 mg/g BW	9.43±0.14*
8 mg/g BW	9.51±0.42*
12 mg/g BW	9.73±0.04*
Aqueous extract:	
4 mg/g BW	9.62±0.22*
8 mg/g BW	9.74±0.24*
12 mg/g BW	9.87±0.12*

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The effect of deer antler from East Kalimantan to increase trabecular bone density and calcium levels serum on osteoporotic mice

[1]Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indoensia, Phone: +6281615886978; E-mail: rr-retno-w@ff.unair.ac.id
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[4]UPTD Pembibitan dan Inseminasi Buatan, Dinas Peternakan dan Kesehatan Hewan Provinsi Kalimantan Timur, Penajam Paser Utara, Indonesia

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Introduction

Osteoporosis is considered to be a skeletal problem due to impaired bone strength that results in an increased risk of fracture [1]. Novel epidemiological studies have shown that osteoporosis becomes a primary public health problem not only for women population, but also for men population. It is estimated that the total amount of hip fractures in women and men in 2025 will be alike [2,3]. In men, the distribution of osteoporosis prevalence is bimodal, showing that the initial peak (<50 year old) is mostly due secondary osteoporosis, while the later peak (>60 year old) is mostly categorized as primary osteoporosis [4]. Correspond to the World Health Organization (WHO), by applying the standard from The International Society for Clinical Densitometry, it is estimated that 1 to 2 million men in the United States have osteoporosis (T-score <-2.5) and 8 to 13 million have osteopenia (T-score between -1.0 and -2.5), or their prevalence are 6% for osteoporosis and 47% for osteopenia [3,5,6]. In aging population, morbidity and mortality from hip fractures are higher in men than in women with fatality rates, among over 75 years is 20.7% in men versus 7.5% in women [7]. The causes of osteoporosis in men are relative to hormonal, genetics, environmental, other specific disease factors, and along 50% of men with secondary osteoporosis [6,8]. The three primary causes of secondary osteoporosis in men are glucocorticoid excess, hypogonadism, and alcohol abuse. The precaution and therapy of osteoporosis disease according to the Recommendation of American College of Rheumatology Ad Hoc Committee is using supplementation with vitamin D and calcium, anti-resorptive agents, calcitonin, gonadal sex hormone replacement, and modifying lifestyle risk factors [9]. Antiresorptive agents that are currently widely used are nitrogenous bisphosphonates which

Retno Widyowati (RW) is the corresponding author[a].

contain alendronate and risedronate, and also calcitonin, estrogen, and raloxifene selective estrogen receptor modulators. These agents increase bone strength and degrade the risk of fractures to varying degrees [10,11]. Some clinical evidences recommend a role for phytoestrogen in the therapy of osteoporosis [12,13,14,15]. Previous studies showed that 70% ethanol and aqueous extracts of deer antler from East Kalimantan increased the alkaline phosphatase (ALP) and mineralization activities of 7F2 cell that is an osteoblast cell lines [16,17]. These extracts contain several major constituents such as protein, lipids, ash, calcium, collagen, chondroitin sulfate and glucosamine [18,19]. Based on these data, it is necessary to prove whether the 70% ethanol and aqueous extracts of deer antler can increase the bone density of osteoporotic mice.

Materials and methods

Materials

Deer antler of *Rusa unicolor* was collected in the middle of March 2017 in UPTD (Technical Implementation Service Unit) of East Kalimantan, Indonesia and voucher specimens were deposited at the UPTD of East Kalimantan, Indonesia. The experimental animal used was male mice (*Mus musculus*) obtained from the Animal Laboratory of the Faculty of Pharmacy, Universitas Airlangga, Surabaya (No.2.KE.176.09.2019). The mice were 5 months old, healthy and weighed of 19.208±10.265 g. The materials were Dexamethasone tablets (Generic, Indonesia), Alendronate® (Novell Pharma, Indonesia), Ethanol pro analysis (Merck, Indonesia), HCl Ketamine (Kepro BV, Indonesia), and CMC-Na.

Extraction of *Rusa unicolor* antlers

Rusa unicolor antler powder was received from UPTD of East Kalimantan, Indonesia. The 991 g of powder was extracted with 70% ethanol – water (2.0 L x 3) using maceration method. The 70% ethanol solution was concentrated using BUCHI rotary evaporator to get 70% ethanol extract (Et-TL, 35.0 g). In addition, the deer antler powder (430 g) was extracted with 100% water (1.0 L x 3) by applying continuous percolation method. The water solution was dried by freeze dried to get aqueous extract (A-TL, 6.1 g).

Trabecular bone density and calcium levels in serum

This study applied a posttest control group design. The 54 healthy male mice were randomly divided into 9 groups. There were healthy control group (without dexamethasone induction), osteoporotic group (induction with dexamethasone and without extracts), positive control group (alendronate suspension), the 70% ethanol extract groups of deer antler (4, 8 & 12 mg/kg BW), and the aqueous extract groups of deer antler (4, 8 & 12 mg/kg BW). First of all, the mice were inducted by 1 mL dexamethasone (0.0029 mg/20 g BW/day) orally for four weeks to obtain osteoporotic conditions [20]. Then the mice were carried out with or without 1 mL extracts orally for four weeks. After 4 weeks, they were sacrificed with anesthesia using HCl ketamine at 10 mg/g BW, i.p. [21] and their femur bones and blood were taken. Blood sampling was performed to measure the calcium levels in serum, whereas femoral bones were taken as the material for histological preparations. The femoral bones were immediately fixed in 10% neutral-buffered formalin and placed in decalcifying solution for 24 hours at 37°C, continuous with being dehydrated and embedded in paraffin. The proximal femur section was stained with a Hematoxylin-eosin (HE) staining. Then, histomorphometry observations of the percentage of trabecular bone density using the Optic Lab microscope and computer software of Image J and Adobe Photoshop were performed [22]. Calculation of trabecular bone density was obtained by dividing the area of observed trabecular bone (μm^2) with the area of entire measurement area (trabecular bone and bone marrow space). The formula for calculating bone density was as follows [23]:

$$\text{Trabecular Bone Density \% BV/TV} = \frac{\text{the area of observed trabecular bone (T)}}{\text{the area of entire measurement area (T+TS)}} \times 100\%$$

The observations were made in the metaphysical region that approached epiphyseal line and it was in cortisol bone region [23]. The bone density values were obtained in units of % BV/TV (% Bone volume/Tissue volume).

Statistical analysis

The experiments were carried out for three more consecutive times using similar sample. It was then presented as means ± standard deviations. The statistical data were performed using SPSS 23 and statistical significance was set at $p<0.05$.

Results

The result of dexamethasone induction for 4 weeks caused osteoporotic occurred in mice due to decreased trabecular bone density and characterized by a change in the vertebrae posture into kyphosis (Fig. 1). Changes in kyphosis posture occurred in all groups (O, A, Et and EA) except the healthy group (H).

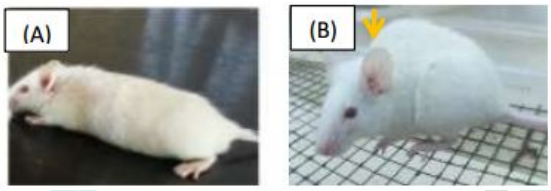

Figure 1. Healthy mice (A) and osteoporotic mice (B), arrow point marks changes in vertebrate posture to kyphosis.

The occurrence of osteoporosis in mice due to dexamethasone induction was not only observed visually, but histomorphometry observation was also carried out on the trabecular area of mice (Fig. 2). It could be seen that the trabecular femur area of osteoporotic group was narrower than healthy group. Then, these results were measured in percentage of trabecular bone density (Fig. 3).

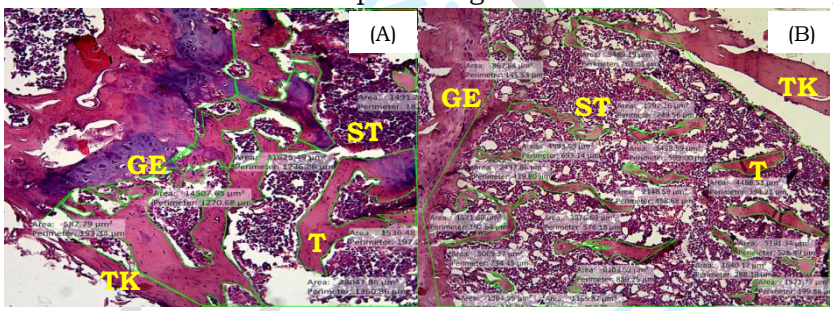

Figure 2. Histomorphometry of the trabecular area from control group (A) and osteoporotic group (B) by hematoxylin-eosin staining. T = Trabecular Bone, ST = Bone Marrow, TK = Cortical Bone, GE = Epiphyseal Line (100x Magnification).

Dexamethasone induction for 4 weeks in mice reduced the trabecular bone density (O) to 23.79±1.23%, while the healthy group (H) was 17.48±1.57% (Fig.3). These results were statistically analyzed using SPSS 23 and the α value was <0.05, it showed that osteoporotic mice group suffered osteoporosis disease.

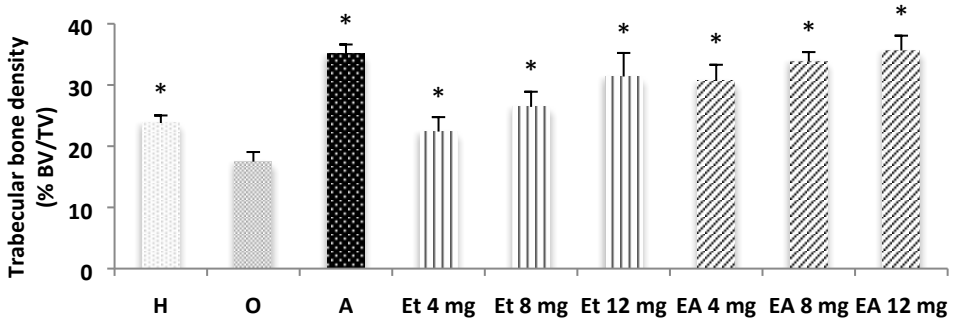

Figure 3. Trabecular bone density levels of several groups; healthy (H), osteoporotic (O), positive control (A, alendronate), 70% ethanol extracts (Et at 4 mg, 8 mg, and 12 mg), and aqueous

extracts (EA at 4 mg, 8 mg, and 12 mg) after intervention, (*p<0.05 compare to osteoporotic group).

Observation of anti-osteoporosis activity test from 70% ethanol and aqueous extracts of deer antlers was done through Histomorphometry calculation of the average trabecular bone density (%). The results showed that trabecular bone density of the osteoporotic, positive control, 70% ethanol extract at 4 mg/kg BW, 8 mg/kg BW, and 12 mg/kg BW and aqueous extract at 4 mg/kg BW, 8 mg/kg BW, and 12 mg/kg BW groups were 17.48±1.57%; 35.09±1.53%; 22.39±2.36%; 26.42±2.47%; 31.83±3.84%; 30.66±2.65%; 33.84±1.53%; and 35.64±2.42% respectively (Fig.3). Furthermore, a statistical analysis test was performed using SPSS 23 (α <0.05) and the results showed that 70% ethanol extract and aqueous from deer antlers at three concentrations had anti-osteoporosis activity by increasing the value of trabecular bone density, significantly, compared to the osteoporotic group.

Table 1. Calcium levels in serum of several groups after intervention (*p<0.05).

Groups	Calcium levels in serum (ppm)
Healthy	9.65±0.02*
Osteoporotic	9.15±0.04
Positive control	9.89±0.05*
70% ethanol extract:	
4 mg/g BW	9.43±0.14*
8 mg/g BW	9.51±0.42*
12 mg/g BW	9.73±0.04*
Aqueous extract:	
4 mg/g BW	9.62±0.22*
8 mg/g BW	9.74±0.24*
12 mg/g BW	9.87±0.12*

Calcium levels in the obtained blood/serum were measured by using a spectrophotometer, as shown in table 1. The results were analyzed using SPSS with a p value of <0.05 and showed that the value of calcium levels in serum increased in the 70% ethanol and aqueous extract of deer antlers at dose dependent manner compared to the osteoporotic group.

Discussion

Deer antlers have been highly used in traditional oriental medicine and several studies have examined the benefits of potential effects. An evaluation of the effect of deer antler on physical growth and bone development is necessary and relevant for a mount of bioactive substances in deer antler, which are assured to have bone-strengthening assets [24]. Therefore, we assessed bone development parameter (femoral bone densitometry) and bone-related enzyme (calcium). Calcium in serum directly affects bone calcification and dissolution and can be measured as parameters related to bone metabolism.

Before anti-osteoporosis activity was carried out, mice were induced by dexamethasone for 14 days so that the mice suffered osteoporosis disease. Dexamethasone is a glucocorticoid drug that can directly inhibit osteoblast activity and inhibit the production of sex hormones that affect bone formation. The use of dexamethasone for a long time (4 weeks at a dose of 0.0029 mg/20 g BW rat/day) causes a decrease in the average percentage of trabecular bone density [20].

The effect of dexamethasone induction in this study has been seen by changing the vertebrae posture into the kyphosis bone, as in Figure 1, and was supported by histomorphometry observations in the trabecular area of mice, as in Figure 2. The figure showed that the trabecular area of osteoporotic group was narrower than the area of healthy group. Then, histomorphometry results were measured in percentage of trabecular bone density, as in Figure 3, that showed that there was a decrease in the percentage of trabecular bone density in osteoporotic group compared with the healthy group. Low total bone density is the causes of osteoporosis and has several cytokines such as IL, 1β-11 and TNFα that stimulate aromatase activity of osteoblast cells [25] and have effect to intestinal metabolism of phyto-testosterone [26]. Ma *et al.* (2011) reported that glucocorticoid had increased the expression and signaling activity of β2-adrenergic receptors in osteoblast. These stimulations inhibited osteoblast proliferation, stimulated osteoclastogenesis and increased regulation of nuclear factor-κB ligand expression [27].

Dexamethasone-induced mice were given a test treatment for 4 weeks, then their calcium levels in serum were observed and histophotometry of their trabecular bone density were examined.

Calcium is a mineral found in bones (99%). In the study of ovariectomy osteoporotic rat models showed that calcium levels in serum decreased to 7.26 mg/dl compared to normal rat, which was 8.35 mg/dl [28]. Glucocorticoid induction can reduce calcium levels in serum by decreasing the absorption of calcium from intestine and inhibiting calcium reabsorption in kidney tubules thereby increasing the excretion of calcium through urine [29].

Based on the statistical analysis, there were significant differences of trabecular bone density between positive control, the 70% ethanol extract and the aqueous extract groups toward osteoporotic group. This study showed that the level of trabecular bone density of osteoporosis group (after intervention with dexamethasone for 4 weeks) was lower than other groups. There was an increase of trabecular bone density in positive control group. There is not too much information regarding the mechanism but it is possible that the alendronate mechanism of action may be indirect. After 12 months of therapy, alendronate was found to stimulate bone mass in femoral neck and lumbar spine in androgen replacing men with long-term hypogonadism. After 6 months of alendronate treatment, urinary deoxypyridinoline which marker of bone resorption was decreased significantly [30]. This condition will result in the balance of bone remodeling and increase osteocalcin as serum marker of bone formation [31].

Shimon et al., (2005) informed that alendronate at 10 mg daily in osteoporotic men with long-standing hypogonadism for 6-12 months increased lumbar-spine bone mineral density significantly ($p<0.005$) [30]. Alendronate is an anti-resoptive agent that hampers farnesyl diphosphate (FPP) synthase, thereby blocking the prenylation of small signalling proteins that is important for osteoclast function and viability [32,33]. Revell (1986) reported that histomorphometry method could be used to show a great correlation between the actual bone volume (0,998) [34].

Several components in deer antlers have started a direct modulation effect on bone growth. Deer antlers have been declared to contain essential amino acids, hyaluronic acid, chondroitin sulfate, collagen, polysaccharides, glycosaminoglics, a number of fatty acids (C18: 3-omega-6 fatty acids), phosphorus, zinc, iron, and calcium [35]. Chondroitin sulfate in deer antlers is a major glycosaminoglycan [36,37] which is water soluble and shows an increased growth effect on osteoblast cells [38] and believed to manage water storage, differentiation, and proliferation of chondrocytes in cartilage tissue.

Overall, the positive effects observed from deer antler extracts from East Kalimantan on bone development in this study are appropiate with some fundamental of traditional Chinese medicine. The proper mechanism of action and the biologically active substances responsible for these effects will involve further research to be explained.

Conclusions

Dexamethasone induction for 4 weeks caused osteoporotic mice, markedly by the occurrence of kyphosis and the narrowing of the trabecular area. The administration of 70% ethanol and aqueous extracts of deer antler from East Kalimantan increased trabecular bone density and calcium levels in dose dependent manner. Thus, the extracts stimulated bone turnover.

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Thu, Sep 10, 2020 at 12:09 PM

10-Sep-2020

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1. The title should be ".....calcium levels in serum ....."
2. The revised Methods in Abstract, "A total of 54 healthy male mice were randomly divided into 9 groups, i.e., healthy control, osteoporotic, positive control, 70% ethanol (4, 8 & 12 mg/kg BW), and aqueous extracts (4, 8 & 12 mg/kg BW) of deer antler groups. All of the interventions were given 1 mL of test sample for 4 weeks orally.
3. In the Discussion, "..... necessary and relevant for the amount of bioactive substances in deer antler....."

Journal of Basic and Clinical Physiology and Pharmacology

Decision Letter (JBCPP.2020.0140.R1)

From: m.horowitz@mail.huji.ac.il

To: rr-retno-w@ff.unair.ac.id

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Subject: JBCPP.2020.0140.R1 - Decision Revise with Minor Modifications

Body: 10-Sep-2020

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Reviewer: 1

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1. The title should be "…….calcium levels in serum ……."
2. The revised Methods in Abstract, "A total of 54 healthy male mice were randomly divided into 9 groups, i.e., healthy control, osteoporotic, positive control, 70% ethanol (4, 8 & 12 mg/kg BW), and aqueous extracts (4, 8 & 12 mg/kg BW) of deer antler groups. All of the interventions were given 1 mL of test sample for 4 weeks orally.
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23-Sep-2020

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Retno Widyowati, PhD

Journal of Basic and Clinical Physiology and Pharmacology

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