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The Effect of Rusa unicolor Antler Extracts from East Kalimantan in Bone Turnover Cell Models

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INTRODUCTION: Osteoporosis is a disease described by a skeletal degradation of bone tissue domination to increase risk of fracture. Traditionally, Rusa unicolor antler from East Kalimantan is used to treat many kinds of diseases, and one of them is disease related to bone turnover. The present research aims to analyze the effects of 70% ethanol and aqueous extracts of Rusa unicolor antler from East Kalimantan on nitric oxide inhibition, osteoblast differentiation, and mineralization related to bone turnover.

METHODS: The nitric oxide inhibition of the extracts in LPS-stimulated RAW 264.7 cells were evaluated by Griess reagent while osteoblast differentiations of extracts were evaluated by measuring alkaline phosphatase in p-nitrophenyl phosphate and their mineralization was determined using Alizarin Red Staining method.

RESULTS: The 70% ethanol and aqueous extracts inhibited cells inflammation (40% and 80%) and stimulated osteoblast differentiation (65% and 52%), respectively. In mineralization test, the aqueous extract showed two times higher effect than that of 70% ethanol extract.

DISCUSSION AND CONCLUSION: The extracts can be considered to successfully reduce expression of inflammatory markers on osteoblasts and maintain osteoblast functions.

Keywords: Antler, Rusa unicolor, alkaline phosphatase, mineralization, NO inhibition

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Message to Editor (Confident to editor)

This article explored bone turnover activity of Rusa unicolor deer, this deer originates from eastern Kalimantan and has never been published.

Thank you very much for reviewed our article and we already revised belong to reviewers comments.

First reviewer:
The paper reported that the effect of Rusa unicolor Antler extracts in bone cell models. Generally, the paper idea is good and suits in the journal. I suggested some corrections before publications.
1. Results section in the abstract should be improved.
Done
2. The authors should be given the prevalence of osteporosis in the World at introduction section.
Done
3. Page 5, line 143, please check. It should be microgram (100 µg/mL)
Thank you for your correction.
4. Page 5, line 149 " calibration curves (which concentration???).
Thank you for your correction, the sensitivity and range value are 2.5 and 20-100 µM
5. Page 6, line 179, please check.
Done, thank you
6. PAge 6, line 184, …." several countries(Reference???).
Done, I already added references from china, taiwan and korea. Thank you
7. The authors should be deleted "from East Kalimantan" in text. This could be just in title, abstract and material section.
Thank you for your suggestion.
8. Page 8, line 241, "They results……..." Please explain how sugar lowering inflammatory respones?
Choi HS et al25 concluded that the sugar content in methanol fraction was significantly 3 times higher than hexane fraction which
contributed to increase in DMSO solubility. Methanol fraction significantly reduced NO production in RAW 264.7 cells as a mediator of
the inflammatory response, but hexane fraction with higher hydrophobicity did not inhibit NO production. These data indicated that
molecules containing hydrophilic portion of deer bone oil inhibit the inflammatory response and correlate with higher sugar levels in
methanol playing an important role in suppressing the inflammatory response by controlling pro-inflammatory cytokines and mediators.
9. Figure 6 should be improved.
Thank you for your suggestion.

Second reviewer:
The manuscript aimed to evaluate the effects of ethanol and aqueous Antler extracts on bone mineralization and no production. There are some minor and major issues to be revised in the article:

- The authors research the effect of Antler extract on both osteoblast differentiation and inflammation in different cell lines. The relation of these parameters must be discussed in detail.
Thank you for your suggestion and the author added explaination the relationship between the two cell lines in this study
- On the other hand, in method section only 7F2 cell line has been mentioned, but there is no info about Raw 264.7 cell line. The authors must explain the reason why they chose this cell lines supplemented with literature.
Done
- In results section, in Figure 1 and figure 3 the statistical significant is not shown.
Done
- The discussion of the results seems weak and must be strengthened.
Thank you very much for your suggestion, I already added several paragraphs to make strength discuss.
- In conclusion, the sentences are not clear. The conclusion of the research should be well defined.
Done and thank you very much, you are right.
- The article should be edited for language. There are lots of mistakes and some sentences are not understandable.
I already revise it and thank you. Hoping your kindness.

The blue color paragraphs are new paragraph that the author added for making more completed statements.

Thank you very much and looking forward for good decision.

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The Effect of *Rusa unicolor* Antler Extracts from East Kalimantan in Bone Turnover Cell Models

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ABSTRACT

Objectives: Osteoporosis is a disease described by a skeletal degradation of bone tissue dominating to increase risk of fracture. Traditionally, *Rusa unicolor* antler from East Kalimantan use to treat many kind of disease, one of them is disease related to bone turnover. The present research was analyzed the effects of 70% ethanol and aqueous extracts of *Rusa unicolor* antler from East Kalimantan on nitric oxide inhibition, osteoblast differentiation and mineralization related to bone turnover.

Materials and methods: The nitric oxide inhibition of the extracts in LPS-stimulated RAW 264.7 cells were evaluated by Griess reagent. While osteoblast differentiations of extracts were evaluated by measuring alkaline phosphatase in p-nitrophenyl phosphate and their mineralization determined by Alizarin Red S method.

Results: The 70% ethanol and aqueous extracts inhibited cells inflammation (40% and 80%) and stimulated osteoblast differentiation (65% and 52%), respectively. In mineralization test, the aqueous extract showed two times higher effect than that of 70% ethanol extract.

Conclusion: The extracts can successfully reduce expression of inflammatory markers on osteoblasts and maintain osteoblast functions.

Keywords: Antler, *Rusa unicolor*, alkaline phosphatase, mineralization, NO inhibition.

INTRODUCTION

Osteoporosis is bone disorder described by a skeletal degradation of bone tissue dominating to an increased risk of fracture and being a silent disease in many complicated situation.[1] It is being important problem of elderly and expected to rise with increased age and life span. At present, 200 million people worldwide were estimated to suffer from osteoporosis.[2] The latest statistical data from the International Osteoporosis Foundation showed that 1 in 3 women over the age of 50 and 1 in 5 men will suffer osteoporosis fractures the rest of their lives.[3] This problem also occurs in Indonesia which has reached a level of caution because the number of osteoporosis sufferer is far greater than the latest data (>19.7%). The number of elderly people in Indonesia is expected to rise by 14% in the period of 1990-2025, while menopausal women in 2000 contributed to an increase of 15.5 million to 24 million in 2015.[4]

For thousands of years, natural plants and animal resources have played a vital role in the development of pharmaceutical drugs and food supplement for the treatment and prevention of diseases.[5] One of such animals with high medicinal value is deer antler which belongs to the Cervidae family. It is an efficient traditional medicine for strengthening bones and tendons. Researchers believe that the imbalance between cartilage erosion and regeneration in people with osteoarthritis is caused by a lack of glycosaminoglycans, these substances has an essential in the cartilage structural integrity. Glycosaminoglycan is isolated from four parts of deer antler (*Cervus elaphus*), such as tip, upper, middle and base through cellulose acetate electrophoresis, enzimatic digestion and chromatography methods. Chondroitin sulfate which contained 88% uronic acid, was the principal glycosaminoglycan. Apart from chondroitin sulfate, deer antler also contains hyaluronic acid, keratan sulfate, and dermatan sulfate in little quantities.[6] Then, some studies showed that deer antler can reduce or even eliminate symptoms associated with osteoarthritis.

For decades, the Chinese people have traditionally used the Deer antler extract to strengthen bones, enhance virility, supplement vitality, feed blood, and enhance male and female sexual organs.[7] A good number of products analogous to deer

antler showed great potential effects on diseases associated with infection, immune dysfunction, and aging. However, the bioactive compounds accountable for this mechanism are unclear.[8-13] According to some preclinical studies, deer antler products reduces animals osteoporosis.[14-16] Lee et al. (2011)[17] showed facilitation of osteoblast proliferation and mineralization are some of the mechanism underlying the effects of deer antler products. Furthermore, Choi et al. (2013)[18] reported an inhibition of osteoclast differentiation by deer antlers. Protein, ash, lipids, collagen, and calcium are some of the chemicals contained in antler.[19] Proteoglycan, testosterone, cholesterol, estradiol, glutamic acid, insulin-like growth factor 1 (IGF-1), iron, aspartic acid, and glycine are also contained in it.[20]

In this study, we used 70% ethanol and aqueous extract of *Rusa unicolor* antler from East Kalimantan, Indonesia, to evaluate the effect of osteoblast differentiation, mineralization and expression of inflammatory markers by measuring alkaline phosphatase (ALP), Alizarin Red Staining (ARS) and nitric oxide (NO) inhibition values, respectively.

MATERIALS AND METHODS

Cell Culture and Reagents

Reagent chemicals such as Alkaline Phosphatase Colorimetric Assay Kit, Acid Phosphatase Leukocyte Kit, and lots more, were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). All cell culture materials and solvents were bought from Thermo Fisher Scientific (Waltham, MA, USA) and analytical grade (J.T. Baker, USA). Macrophages (RAW 264.7) and mouse osteoblast-like cells (7F2) were purchased from Food Industry Research and Development Institute, Taiwan, and refined in Dulbecco's Modified Eagle's Medium (DMEM). They are further strengthened with 10% v/v Fetal Bovine Serum (FBS), 100 µg/mL streptomycin, and 100 units/mL penicillin. Cells were refined in a dabbled incubator with 5% CO_2 at 37°C.

Antler materials

Deer antler of *Rusa unicolor* was collected in middle March 2017 in UPTD East Kalimantan, Indonesia, and voucher specimens were deposited at the UPTD East Kalimantan, Indonesia.

Extraction of Rusa unicolor Antlers

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

Cell Viability Assay

The RAW 264.7 cells were plated for cell growth studies at a density of 5 x 10^4 cells/well in 96-well plates. DMEM medium consisting of 100 units/mL penicillin, 10% FBS and 100 µg/mL streptomycin was used to restore the cell. After 24 hours, Et-TL and A-TL extracts of *Rusa unicolor* antler were incubated at various concentrations (10, 50 and 100 µg/mL) for another 24 hours at 37°C. The cell supernatants were subsequently extracted, after 200 µL 3-(4,5-dimethylthiazol-2-yl)- and 100 µL 2,5-Diphenyltetrazolium Bromide (MTT) reagent (100 µg/mL) were incubated for 4 hours. Similarly, to dissolve the formazan crystals, 100 µL dimethyl sulfoxide (DMSO) was added. The absorbance was ruminated at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader. All experiments were carried out in triplicate, with the relative cell viability (%) declared as a portion relative to the unprocessed control cells. The same method was carried out on 7F2 osteoblast-like cells (10^4 cells/well) in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin.[21]

Nitrite Measurement

The 24-well plates were used to seed the RAW 264.7 cells (5×10^5 cells/well) with DMEM containing 100 unit/mL penicillin, 10% FBS, and 100 µg/mL streptomycin with a 24 hours incubation duration at 37°C. The cells were aroused with 500 ng/mL lipopolysaccharide (LPS) and examined with Et-TL and A-TL extracts at 10–100 µg/mL for 1 day. The emitted NO was estimated by weighing the nitrite concentration. The 100 µL Et-TL and A-TL extracts were added with 100 µL of Griess reagent in 96-well plates for 15 minutes at room temperature. ELISA reader was used to deliberate the absorbance at 550 nm. Furthermore, standard calibration

curves were processed using sodium nitrite to evaluate nitrite expression from cells with sensitivity and linear range values of 2.5 µM and 20-100 µM, respectively.[21]

Differentiation of Cellular Alkaline Phosphatase Activity (ALP)

The 7F2 osteoblast-like cells were cultured in 24-well plates at a density of 10^4 in DMEM containing 5 mM β-glycerol phosphate (β-GP), 10% FBS and 50 µg/mL ascorbic acid with or without 10–100 µg/mL Et-TL and A-TL extracts with a 4 days incubation period at 37°C in a 5% CO_2 atmosphere. Phosphate buffer saline (PBS) was used to wash the extracted supernatants. After that, a percentage of v/v triton solution was inserted and incubated for 10 minutes at 37°C. After incubation, the cell lysates were examined for ALP by adding 200 µL p-nitrophenyl phosphate (PNPP) and di-ethanolamine buffer into each well for a period of 30 minutes and at room temperature. The 50 µL/well stop solution was inserted to end the reaction while ELISA reader at 405 nm was used to evaluate the absorbance.[21]

Mineralization of Extracellular Matrix

One mL DMEM comprising of 10% FBS, 5 mM β-GP and 50 µg/mL ascorbic acid (2GF medium) was used to seed a portion the osteoblast-like cells at a density of 10^4 cells/well in an incubator for 7 days. The Et-TL and A-TL extracts were also added to the growth medium at concentration of 10 – 100 µg/mL. PBS and 75% v/v ethanol were used to wash the samples and then dried at room temperature. The cells were stained with 1% ARS (200 µL) for one hour. A microscope (Nikon TI-E) and SPOT RT3 camera were used to note the images of the cell morphology. Each well received 400 µL of 10% w/v cetylpyridinium chloride and shaken for 10 minutes in order to dissolve the calcium. The absorbance was finally evaluated using an ELISA reader at 560 nm.[21]

Statistical Analysis

The experiments were carried out for three more consecutive times using similar results. It then presented as means ± standard deviations. Paired t-test was used to illustrate data analysis. The differences proved to be statistically significant at $P<0.05$.

RESULTS

The Effect of Rusa unicolor Antler Extract on Cell Viability and NO Production

In this research, we analyzed the effects of Et-TL and A-TL extracts toward anti-inflammatory related bone turnover. Several research reported on the pharmacological effects of deer bones and antlers from several countries, but there have been no reports on deer antlers from Indonesia, *Rusa unicolor*.[7,22-24]

In vitro cytotoxic samples were examined using MTT test in RAW 264.7 cells. Samples (Et-TL and A-TL extracts) with different concentrations were used to incubate cells for 24 hours and examined by MTT method. After 24 hours, data was represented as the mean cell viability. The A-TL extract did not show cytotoxicity compare to Et-TL extracts (Fig. 1). The cell viability significantly decreased in Et-TL extracts (50 and 100 µg/mL). The result showed that the aqueous extract was not toxic toward the RAW 264.7 cells at the concentration range of 10–100 µg/mL. It could also decrease cytotoxicity and increase absorption of cellular uptake of compounds in site.

The anti-inflammatory activity of samples in LPS-stimulated RAW 264.7 cells was conducted by observing the NO inhibition. The Griess reagent was used to evaluate the nitrite value that is directly related to the amount of NO production using RAW 264.7 cells. These cells were incubated with samples, after which the NO produced in the LPS-stimulated RAW 264.7 cells deteriorated significantly (Fig. 2). The Et-TL extract stopped NO production until it was at 40% in 10 µg/mL. Similarly, A-TL extract inhibited until 80% in the same concentration. LPS was not used on the cells of the control group. The total nitrite was manufactured by LPS group (500 ng/mL). This group had a value of 100%.

Effect on ALP Stimulation of 7F2 Osteoblasts of 70% Ethanol and Aqueous Extracts of Deer Antler

The effect of samples (Et-TL and A-TL extracts) in 7F2 osteoblastic cell line proliferation was carried out using MTT test. The viability cells in Et-TL extract decreased significantly (50 and 100 µg/mL) but the value was more than 100%. It showed that high concentration of Et-TL extract was not toxic even though the cell proliferation value decreased. Whereas, cells viability of A-TL extract increased significantly (Fig. 3). As a result, all deer antler extracts decreased cytotoxicity and raised cellular uptake. The ALP and mineralization experiments continued.

The ALP stimulation of 7F2 osteoblast cells using Et-TL and A-TL extracts of deer antler was incubated for 4 days. The effects of test samples on the ALP assay increased in the 7F2 osteoblasts opposed to the 2GF group. After 4 days, the A-TL extract stimulated their ALP activity to 65%, while the Et-TL was stimulated to 52% (Fig. 4).

Effect on Mineralization of 7F2 Osteoblasts of 70% Ethanol and Aqueous Extracts of Deer Antler

The 7F2 osteoblast cells were cultured in 2GF medium to prompt osteoblast differentiation and mineralization. After 7 days of incubations, the effects of Et-TL and A-TL extracts of deer antler on osteoblast mineralization were examined using ARS, which determined the calcium content in the bone matrix. The Et-TL extract displayed the stimulation on cell differentiation (Fig. 4) and after 7 days, this extract stimulated the mineralization. The A-TL extract displayed the best stimulation mineralization than 70% ethanol extract of deer antler (Fig. 5).

The stains on ARS represented mineral deposits and quantified by cetylpyridinium chloride extraction method. The treated 7F2 osteoblast cells increased the ARS accumulation compared to the 2GF group. Photographs were used to represent mineralization process of osteoblast cells under bright field. Several mineralized nodules formation (red) was high in 7F2 osteoblast cells treated with Et-TL and A-TL extracts on the 7^{th} day under microscope observation (Fig. 6). Identical patterns were also observed in ALP activity.

DISCUSSION

Bone remodeling is a process created between bone resorption (osteoclast activity) and formation (osteoblast activity). Osteoclasts are multinucleated cells produced from macrophage precursor cells and osteoclast formation that requires RANKL as receptor activator. The RAW 264.7 macrophages have been shown to play an important role in osteoclast formation and function.[25] Osteoclast function can be impaired due to degeneration of articular cartilage and synovial inflammation involving chemokines, interleukin (IL)-1β, IL-6, IL-11, IL-17, tumor necrosis factor-α (TNF-α) and a number of inflammatory cytokines. They produce loss bone by reducing osteoprotegerin (OPG) production and stimulating receptor activator of nuclear factor kappa-B ligand (RANKL) expression in stromal cells and osteoblasts.

The NO inhibition of RAW 264.7 macrophages showed a decrease in cytokine inflammation thus preventing bone loss by increasing the a mount of OPG and RANKL.[21]

The NO inhibition through inhibition of the JNK, p38, and NF-κB signaling pathways is associated with inflammatory response inhibition and osteoclastogenesis suppression. Yeh et al.[21] showed that Cur liposomes, as sample, inhibited NO production in RAW 264.7 macrophages and prevented osteoclast differentiation by decreasing cathepsin K regulation, tartrate-resistant acid phosphatase (TRAP) expression and increasing OPG/RANKL ratio. The high ratio of OPG/RANKL and NO inhibition caused decrease in osteoclast activation and an increase in the number of osteoblasts, so the osteoporosis effect can be reduced.

In this study, we evaluated NO production of 70% ethanol and aqueous extracts in LPS-stimulated RAW 264.7 macrophages. LPS markedly stimulated NO production in these macrophages compared to its effects in control group. The 70% ethanol extract of deer antler showed higher reaction rate in lowering LPS-stimulated RAW 264.7 than that of aqueous extract (Fig.2). Both of them did not have any cytotoxic effect on RAW 264.7 macrophages (Fig.1). According to Choi et al. (2016),[26] the ability of deer bone methanol fraction in reducing NO production was a result of decreased regulation and expression mRNA from pro-inflammatory mediators, such as cyclooxygenase (COX-2), IL-12β, and IL-1β. The methanol fraction containing sugar played an essential role in lowering inflammatory responses by controlling pro-inflammatory cytokines and mediators. Exorbitant production of pro-inflammatory cytokines has been discovered in variety of diseases, such as cancer, arthritis, rheumatoid arthritis, osteoarthritis and osteoporosis.[27,28]

The osteoblast phenotype for bone mineralization is obtained in two stages. First stage, the mature matrix and specific protein associated with the bone cell phenotype (ALP) is detected. Second stage, the matrix becomes mineralized by calcium deposition. Then, a sponge bone layer is formed around the original cartilage and the space between the sponge bones is filled with a bone matrix and becomes a compact bone.

Antler increased the proliferation of osteoblasts and bone matrix proteins, such as collagen type I and bone sialoprotein (BSP). These increases triggered osteoblast differentiation, such as mineralized nodule formation. As expected, the 70% ethanol and aqueous extracts of deer antler increased ALP activity constantly (Fig.4). The

cells cultured in the medium containing aqueous extract had higher ALP activity than 70% ethanol extract which is similar to the results obtained by Lee et al. (2011).[29] Deer antler increased osteoblasts proliferation significantly up to 119% of the basal value. Significant increased in mRNA expression and ALP activity were found at 50-100 µg/mL. It also increased expression levels of type I collagen mRNA and mineralization more than 183%.[29]

ALP has been proven as an important enzyme in the mineralization process. Human inherited bone abnormalities, hypophosphaphia, can occur due to the absence of expression of ALP bone form[30,31] and was characterized by the absence of mineral deposition in bone (rickets). ALP is important for mineralization but its role has not been fully clarified. We continued to evaluate the effect of 70% ethanol and aqueous extracts of deer antler in mineralization activity (Fig.5).

Bone formation implicated the proliferation, differentiation and mineralization of osteoblasts. The 7F2 osteoblast cells started differentiating at the 4th day. The ALP activity is displayed as a marker of the initial period of cell differentiation. Then, the mineralization of 7F2 osteoblast cells produced by ARS was monitored. Figure 6 showed that the extracts stimulated the mineralization of 7F2 osteoblast cells. The stimulation of aqueous extract at 100 µg/mL was two times higher than that of 70% ethanol extract. This difference may be as a result of reduction of deer antler extracts toxicity or chemical constituents inside both extracts.

CONCLUSION

Both of 70% ethanol and aqueous extracts of *Rusa unicolor* antler from Kalimantan had a role in bone remodeling. The aqueous extract stimulated higher bone differentiation and mineralization than 70% ethanol extract with ALP value of 65% and twice ARS value. Whereas the 70% ethanol extract showed higher NO inhibition activity than aqueous extract with inhibition value of 40%. The extracts can successfully reduce expression of inflammatory markers on osteoblasts and maintain osteoblast functions.

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Conflict of Interest: No conflict of interest was declared by the authors.

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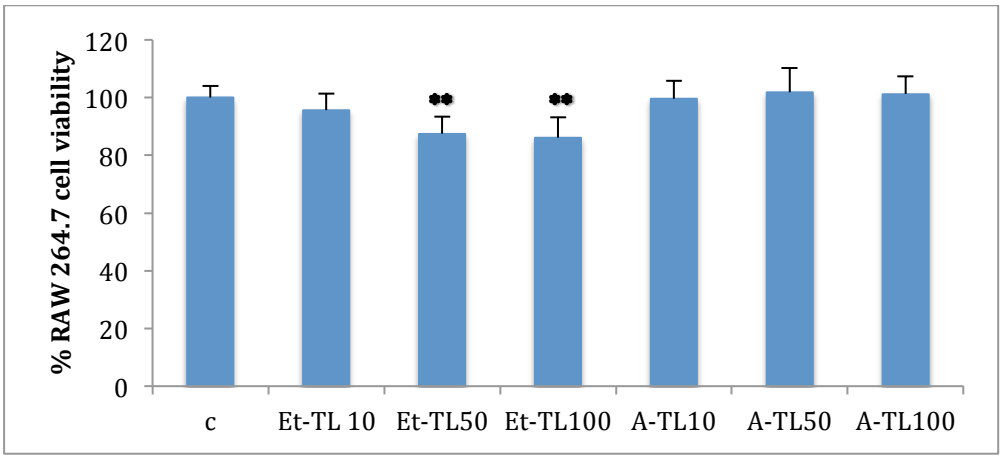

Figure 1. The effects of 70% ethanol extract (Et-TL) and aqueous extract (A-TL) of deer antler from East Kalimantan at 10, 50 and 100 µg/mL on RAW 264.7 cell viability; C was medium without sample. Data are expressed as the mean of percent cell viability compared to control after exposure for 24 hours ± standard deviation.

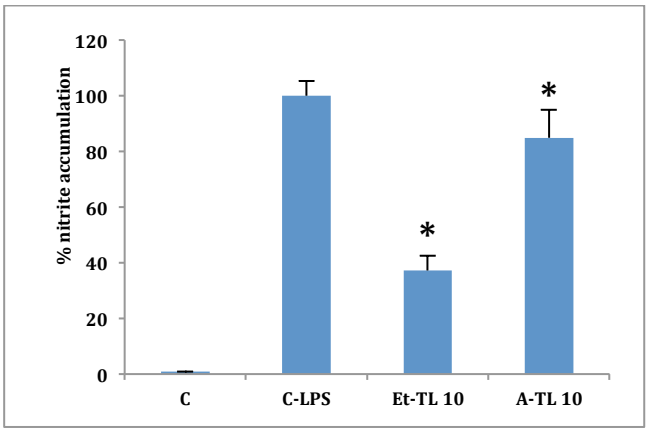

Figure 2. Effects of 70% ethanol extract (Et-TL) and aqueous extract (A-TL) of deer antler from East Kalimantan at 10 µg/mL on NO production in LPS (500 ng/mL)-induced RAW 264.7 macrophages for 24 hours. C-LPS: control of LPS, LPS: lipopolysaccharide, NO: nitric oxide, C: cells of the control group were not induced with LPS. The total nitrite produced by the cells of the LPS group (with the treatment of 500 ng/mL LPS) is expressed as 100%. Results are expressed as percentage with mean ± standard deviation. *P<0.05 with respect to control.

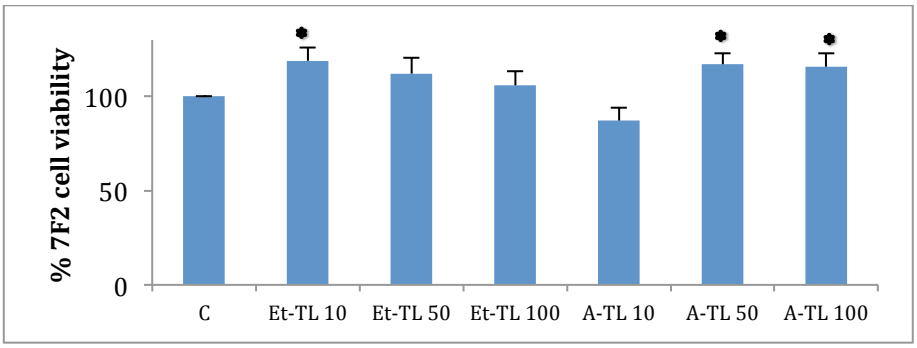

Figure 3. The effects of 70% ethanol extract (Et-TL) and aqueous extract (A-TL) of deer antler from East Kalimantan at 10, 50 and 100 µg/mL on 7F2 cell viability; C was medium without sample. Data are expressed as the mean of percent cell viability compared to control after exposure for 24 hours ± standard deviation.

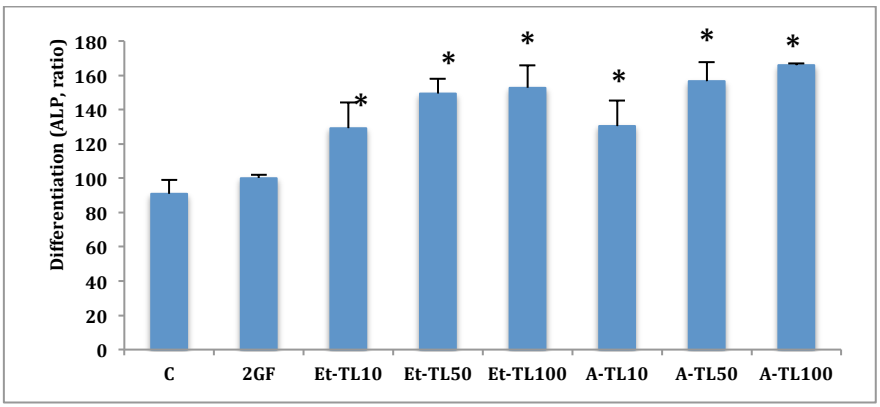

Figure 4. The effects of 70% ethanol extract (Et-TL) and aqueous extract (A-TL) of deer antler from East Kalimantan 10, 50 and 100 µg/mL on osteoblast differentiation (ALP) during 4 days incubations. The 7F2 osteoblast cells were cultured in 2GF (50 µg/mL ascorbic acid and 5 mM β-GP) to induce osteoblast differentiation. Results are expressed as percentage with mean ± standard deviation. *P<0.05 with respect to 2GF.

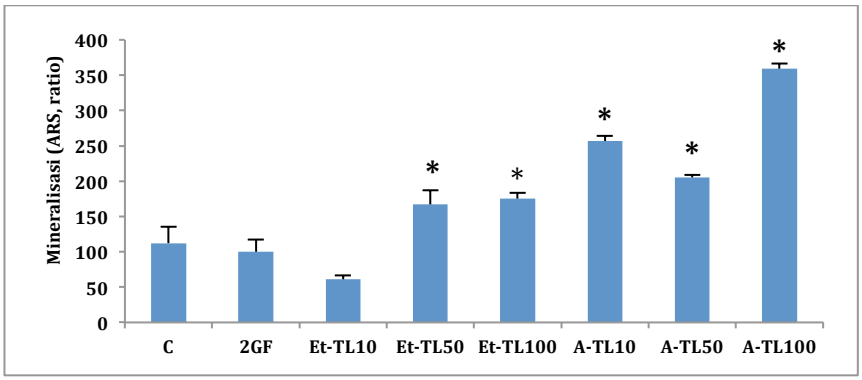

Figure 5. The effects of 70% ethanol extract (Et-TL) and aqueous extract (A-TL) of deer antler from East Kalimantan 10, 50 and 100 µg/mL on quantification mineralization assay during 7 days incubations. ARS stain was quantified by cetylpyridinium chloride extraction method. Results are expressed as percentage with mean ± standard deviation. *P<0.05 with respect to 2GF.

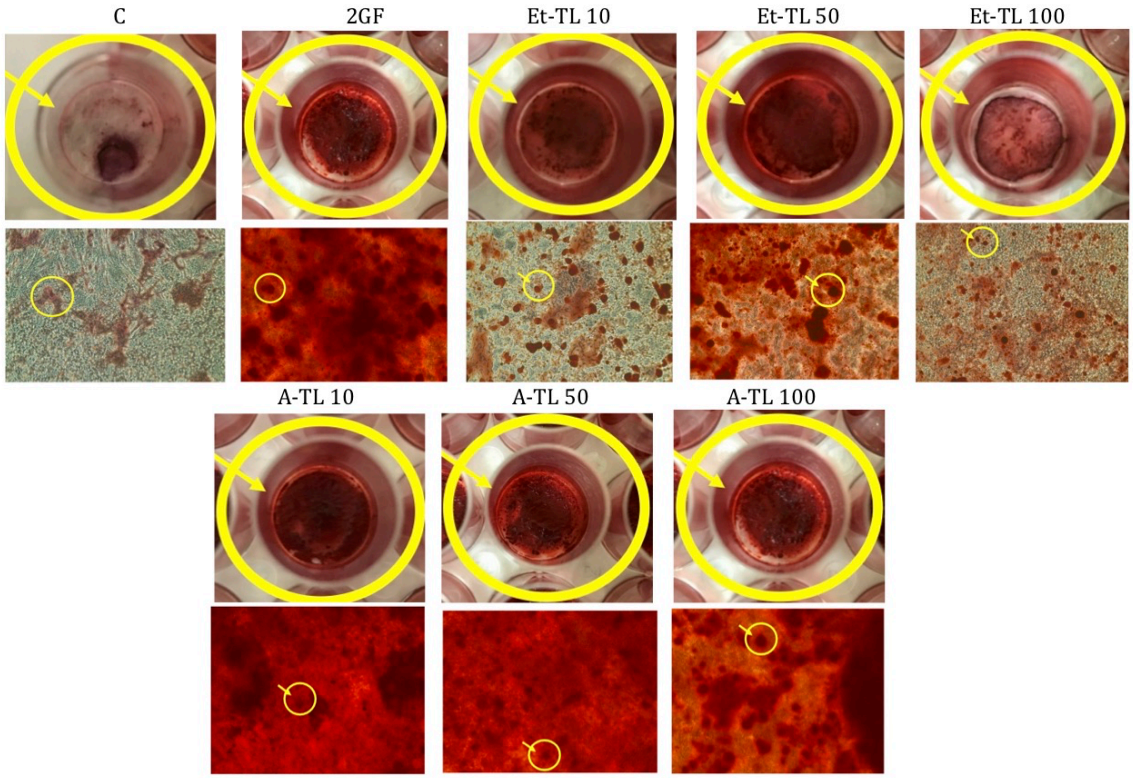

Figure 6. Histochemical staining of mineral deposition of 70% ethanol extract (Et-TL) and aqueous extract (A-TL) of deer antler from East Kalimantan 10, 50 and 100 µg/mL. They were visualized using ARS staining (×100 magnification). Red staining represents mineral deposition.

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