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Submission date: 03-Apr-2023 03:51PM (UTC+0800)

Submission ID: 2054425380

File name: Published_tanduk_rusa.pdf (951.22K)

Word count: 4664

Character count: 25370



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Doğu Kalimantan'dan *Rusa unicolor* Geyiği Boynuzu Ekstraktlarının Kemik Dönüşümü Hücre Modelleri Üzerine Etkisi

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ABSTRACT

Objectives: Osteoporosis is a condition characterized by skeletal degradation of osseous tissue resulting in an escalated chance of broken bones. Traditionally, *Rusa unicolor* horn from East Kalimantan is used to treat many diseases, including conditions associated with bone turnover. The aim of the present research was to analyze the effects of 70% ethanol and aqueous extracts of *R. unicolor* antler's horn from East Kalimantan on nitric oxide inhibition, osteoblast differentiation, and mineralization related to bone turnover.

Materials and Methods: Nitric oxide inhibition of the extracts in lipopolysaccharide-stimulated RAW 264.7 macrophages was evaluated by Griess reagent, while the effects of extracts on osteoblast differentiations were measured by alkaline phosphatase in *p*-nitrophenyl phosphate. Their effects on mineralization was determined using alizarin red staining.

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

Conclusion: The extracts can effectively degrade inflammatory marker expression and preserve osteoblast functions.

Key words: *Rusa unicolor*, antler, alkaline phosphatase, mineralization, nitric oxide inhibition

ÖZ

Amaç: Osteoporoz artmış kemik kırılmalarıyla sonuçlanan kemik dokusundaki iskelet degradesyonu ile karakterize bir durumdur. Geleneksel olarak, Doğu Kalimantan'dan *Rusa unicolor* boynuzu kemik dönüşümü dahil, birçok hastalığın tedavisinde kullanılır. Bu çalışmanın amacı Doğu Kalimantan *R. unicolor* geyiği boynuzunun %70'lik etanol ve sulu ekstraktlarının nitrik oksit inhibisyonu, osteoblast farklılaşması ve kemik dönüşümüyle ilişkili mineralizasyon üzerine etkilerinin araştırılmasıdır.

Gereç ve Yöntemler: Ekstratların lipopolisakkarit ile stimüle edilen RAW 264,7 makrofajlarında nitrik oksit inhibisyonu Griess belirteciyle değerlendirilirken, ekstraktların osteoblast farklılaşması üzerine etkileri *p*-nitrofenil fosfata alkalik fosfataz ölçülerek belirlenmiştir. Mineralizasyon üzerine etkileri ise, alizarin kırmızısı kullanılarak belirlenmiştir.

Bulgular: %70'lik etanol ve sulu ekstraktlarının hücre enflamasyonunu inhibe ettiği (sırasıyla %40 ve %80) ve osteoblast farklılaşmasını stimüle ettiği (sırasıyla %65 ve %52) belirlenmiştir. Mineralizasyon testinde, sulu ekstre etanol ekstresine göre iki kat daha yüksek etki göstermiştir.

Sonuç: Ekstrelerin etkin bir şekilde enflamasyon belirteci ekspresyonunun degrades etmiş ve osteoblast işlevlerini korumaktadır.

Anahtar kelimeler: *Rusa unicolor*, geyik, alkalik fosfataz, mineralizasyon, nitrik oksit inhibisyonu

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Received: 18.01.2019, Accepted: 30.05.2019

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10 INTRODUCTION

Osteoporosis is a bone condition characterized by skeletal degradation of osseous tissue leading to an increased chance of broken bones.¹ It is a condition of old age and supposed to increase with rising age and lifespan. At present, more than 200 million people worldwide are estimated to suffer from osteoporosis.² The latest statistical data from the International Osteoporosis Foundation showed that one in three women above 50 years old and one in five men shall suffer osteoporosis for the rest of their lives.³ Indonesia also has the same problem, whereas the number of osteoporosis sufferers is higher than the latest data (>19.7%). In Indonesia, the number of old people is supposed to increase by 14% in 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 supplements for the treatment and prevention of diseases.⁵ One of such resources with great medicinal value is the antler of the deer belonging to the family Cervidae. It is an efficient traditional medicine for strengthening bones and tendons. Researchers assume that the lack of glycosaminoglycans caused disproportion between cartilage erosion and regeneration in osteoarthritis sufferers. These substances have an essential role in cartilage's structural integrity. Glycosaminoglycan is isolated from four parts of the antler of deer (*Cervus elaphus*), namely the tip, upper part, middle, and base, through cellulose acetate electrophoresis, enzymatic digestion, and chromatography. Chondroitin sulfate, which contains 88% uronic acid, is the principal glycosaminoglycan. Apart from chondroitin sulfate, deer antler also contains hyaluronic acid, dermatan sulfate, and keratan sulfate in small quantities.⁶ Several researches also report that deer antler can reduce or even eradicate symptoms related to osteoarthritis.

For decades, Chinese people have traditionally used deer antler extract to strengthen bones, enhance virility, feed blood, supplement vitality, and enhance male and female sexual organs.⁷ A good number of products analogous to deer antler show great potency on illnesses related to aging, infection, and immune dysfunction. However, the active constituents responsible for this mechanism are unclear.⁸⁻¹³ According to some preclinical studies, deer antler products are able to reduce animals osteoporosis.¹⁴⁻¹⁶ Lee et al.¹⁷ showed that facilitation of osteoblast proliferation and mineralization are some of the principal mechanisms of the effects of deer antler products. Furthermore, Choi et al.¹⁸ reported inhibition of osteoclast differentiation by deer antlers. Protein, calcium, lipids, ash, collagen, proteoglycan, testosterone, cholesterol, estradiol, glutamic acid, insulin-like growth factor 1, iron, aspartic acid, and glycine are some of the constituents contained in antler.^{19,20}

In the present study, we evaluated 70% ethanol and aqueous extract of *Rusa unicolor* antler from East Kalimantan Indonesia toward 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

Chemicals such as an acid phosphatase leukocyte kit and ALP colorimetric assay kit were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Cell culture substances and solvents were bought from Thermo Fisher Scientific (Waltham, MA, USA) and were analytical grade (J.T. Baker, USA). Macrophages (RAW 264.7) and osteoblast cells (7F2) were obtained from the Food Industry Research and Development Institute, Taiwan, and refined in Dulbecco's Modified Eagle's Medium (DMEM). They were further strengthened with 10% v/v fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were refined in an incubator with 5% CO₂ at 37°C.

Antler materials

Deer antler of *R. 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.

R. unicolor antler extraction

R. unicolor antler was obtained from UPTD of East Kalimantan, Indonesia. It was powdered (991 g) and extracted with 70% ethanol-aqueous (2.0 Lx3) using maceration. The 70% ethanol solution was concentrated using a rotary evaporator to obtain 70% ethanol extract (Et-TL, 35.0 g). In addition, the deer antler (430 g) was extracted with 100% water (1.0 Lx3) by applying continuous percolation. The aqueous solution was freeze dried to obtain aqueous extract (A-TL, 6.1 g).

34 Cell viability assay

The RAW 264.7 macrophages were cultured at a density of 5x10⁴ macrophages/well in 96-well plates using DMEM consisting of 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin for macrophage growth studies. After 24 h, the samples (Et-TL and A-TL extracts of *R. unicolor* antler) were incubated at 10, 50, and 100 µg/mL for another 24 h. Afterwards, the medium was discarded and the macrophages were incubated with 200 µL of 3-(4,5-dimethylthiazol-2-yl)- and 100 µL of 2,5-diphenyltetrazolium bromide (MTT) reagents (100 µg/mL) for 4 h. Then 100 µL of dimethyl sulfoxide was added to disperse the formazan crystals. The absorbance was determined at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader. All treatments were carried out in triplicate and expressed in % macrophage viability of unprocessed control macrophages. A similar method was carried out on 7F2 osteoblast-like cells at 10⁴ cells/well in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin.²¹

4 Nitrite measurement

The 5x10⁵ cells/well of RAW 264.7 macrophages were seed in 24-well plates and incubated with DMEM containing 10% FBS, 100 unit/mL penicillin, and 100 µg/mL streptomycin for 24 h. The macrophages were activated with 500 ng/mL lipopolysaccharide (LPS) and evaluated with samples (Et-TL and A-TL extracts) at 10-100 µg/mL for 1 day. The emitted NO was estimated by weighing the nitrite concentration. Then 100

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 μL of samples were added with 100 μL of Griess reagent to 96-well plates for 15 min at room temperature. An ELISA reader was used to measure the absorbance at 550 nm. Furthermore, curves of standard calibration were processed using NaNO_2 to evaluate nitrite expression of macrophages with sensitivity and linear range values of 2.5 μM and 20-100 μM , respectively.²¹

Differentiation of alkaline phosphatase activity

The 10^4 cells/well of 7F2 osteoblast cells were plated in 24-well plates using DMEM containing 10% FBS, 50 $\mu\text{g}/\text{mL}$ ascorbic acid, and 5 mM β -glycerol phosphate (β -GP) with or without 10-100 $\mu\text{g}/\text{mL}$ Et-TL and A-TL extracts for 4 days of incubation (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 added, followed by incubation for 10 min at 37°C. For the ALP assay, the cell lysates were added with 200 μL of *p*-nitrophenyl phosphate and di-ethanolamine buffer to each well for 30 min at room temperature. Then 50 $\mu\text{L}/\text{well}$ NaOH solution was added to end the reaction, while an ELISA reader at 405 nm was used to evaluate the absorbance.²¹

Mineralization of the extracellular matrix

One milliliter of DMEM comprising 5 mM β -GP, 50 $\mu\text{g}/\text{mL}$ ascorbic acid (2GF medium), and 10% FBS was used to seed 10^4 cells/well of 7F2 osteoblast cells for 7 days in an incubator; 10-100 $\mu\text{g}/\text{mL}$ of Et-TL and A-TL extracts were also added to the growth medium. After 7 days, PBS and 75% v/v ethanol were used to wash the samples and they were dried at room temperature. The cells were dyed with 200 μL of 1% ARS for 1 h. A Nikon TI-E microscope and SPOT RT3 camera were used to record images of the cell morphology. Each well received 10% w/v cetylpyridinium chloride (400 μL) and was rocked for 10 min in order to disperse the calcium. The absorbance was finally evaluated at 560 nm using an ELISA reader.²¹

Statistical analysis

The experiments were carried out for three more consecutive times with similar sample. They are presented as mean \pm standard deviations. The paired t-test was used for the data analysis. The differences were significant at $p < 0.05$.

RESULTS

The effect of antler extract on cell viability and nitric oxide production

In the present research, the effects of Et-TL and A-TL extracts against inflammation associated with bone turnover were analyzed. Several researchers have reported the bioactivity impacts of deer bones and antlers from various nations, but there is no information on the pharmacological activity of deer antlers originating from Indonesia (*R. unicolor*).^{7,22-24}

Cytotoxic test samples (Et-TL and A-TL extracts) at different concentrations were incubated with RAW 264.7 macrophages for 1 day and were evaluated using the MTT method. Then the data were represented as the average cell viability. The A-TL extract did not show cytotoxicity compared to Et-TL

extracts (Figure 1), while the macrophage viability in Et-TL extracts (50 and 100 $\mu\text{g}/\text{mL}$) decreased significantly. Thus, the aqueous extract was not toxic toward the macrophages at the concentration series of 10-100 $\mu\text{g}/\text{mL}$ and was able to increase the cellular permeation of constituents therein.²⁴

The anti-inflammatory test of samples in RAW 264.7 macrophages stimulated by LPS was conducted by monitoring the NO inhibition. The Griess reagent was used to evaluate the nitrite value directly related to the amount of NO production in RAW 264.7 macrophages. These macrophages were treated with samples, after which the NO produced in the LPS-stimulated RAW 264.7 macrophages deteriorated significantly (Figure 2). The Et-TL extract prevented NO production up to 40% in 10 $\mu\text{g}/\text{mL}$. Similarly, A-TL extract reduced it up to 80% in the same concentration. LPS (500 ng/mL) in macrophages was not used as a control group because it had a total nitrite value of 100%.

The 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) on 7F2 osteoblastic cell line proliferation was determined by MTT test. The viability of cells in Et-TL extract decreased significantly (50

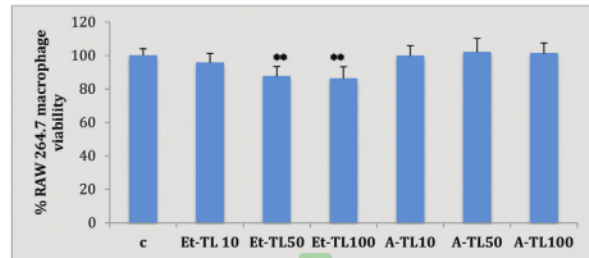


Figure 1. The effects of samples at 10, 50, and 100 $\mu\text{g}/\text{mL}$ on RAW 264.7 macrophage viability; C was the control medium without samples, Et-TL was 70% ethanol extract, A-TL was aqueous extract. Data are shown as the average of % cell viability to control for 24 h \pm standard deviation

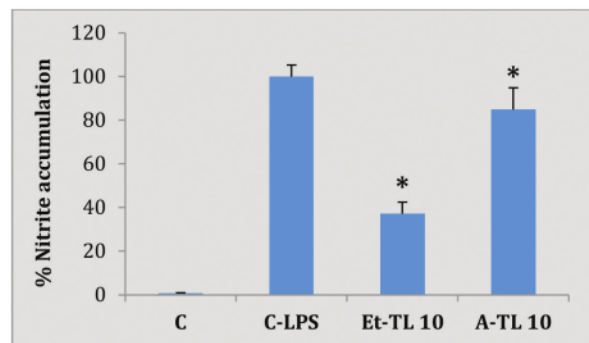


Figure 2. The effects of samples at 10 $\mu\text{g}/\text{mL}$ on NO production in LPS (500 ng/mL)-induced RAW 264.7 macrophages for 24 h; C-LPS was control of LPS, C was cells of the control group were not induced with LPS, Et-TL was 70% ethanol extract, A-TL was aqueous extract. The total nitrite produced by the cells of the LPS group is revealed as 100%. Results are shown as % of average \pm standard deviation. * $p < 0.05$ with respect to control
 NO: Nitric oxide LPS: Lipopolysaccharide

and 100 µg/mL), but the value was more than 100%. It showed that a high concentration of Et-TL extract was not toxic even though the cell proliferation value decreased. On the other hand, cell viability of A-TL extract increased significantly (Figure 3). As a result, all deer antler extracts decreased cytotoxicity and raised cellular uptake. Then the ALP and mineralization experiments continued.

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

The 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 incubation, 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 stimulation of cell differentiation (Figure 4) and after 7 days this extract stimulated mineralization. The A-TL extract displayed better stimulation mineralization than 70% ethanol extract of deer antler (Figure 5).

The stains on ARS represented mineral deposits and were quantified by cetylpyridinium chloride extraction. The treated 7F2 osteoblast cells increased the ARS accumulation compared to the 2GF group. Photographs were used to represent the

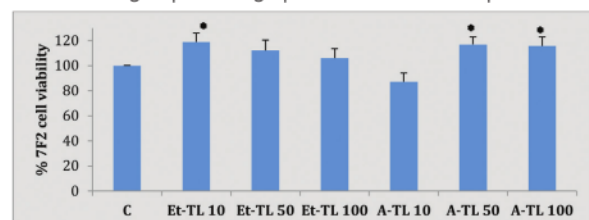


Figure 3. The effects of samples at 10, 50, and 100 µg/mL on 7F2 cell viability; C was medium without sample, Et-TL was 70% ethanol extract, A-TL was aqueous extract. Data are shown as the average of % cell viability compared to control for 24 h ± standard deviation

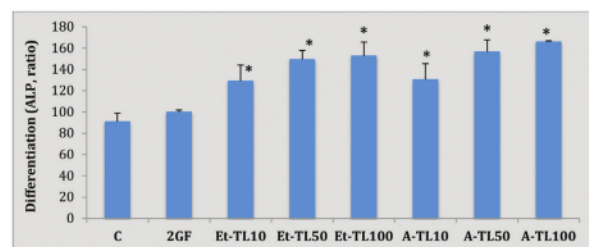


Figure 4. The effects of samples at 10, 50, and 100 µg/mL on osteoblast differentiation (ALP) during 4 days of incubation; Et-TL was 70% ethanol extract, A-TL was aqueous extract. The 7F2 osteoblast cells were cultured in 2GF medium to produce osteoblast differentiation. Results are shown as % with average ± standard deviation. *p<0.05 with respect to 2GF
ALP: Alkaline phosphatase

mineralization process of osteoblast cells under a bright field. Mineralized nodule formation (red) was high in 7F2 osteoblast cells treated with Et-TL and A-TL extracts on day 7 under microscope observation (Figure 6). Identical patterns were also observed in ALP activity.

DISCUSSION

Bone metabolism is a lifelong process that occurs because mature bone tissue is lost (osteoclast activity) and new bone tissue is formed (osteoblast activity). Osteoclasts are multinucleated cells and involve nuclear factor kappa-B ligand (RANKL) as a stimulus of receptors and generated by macrophage precursor cells. The RAW 264.7 macrophages have a necessary role in the process of osteoclast formation and function.²⁵ Osteoclast function can be impaired due to degeneration of articular cartilage and synovial inflammation involving tumor necrosis factor- α , chemokines, and a number of inflammatory cytokines and interleukins (IL) such as IL-1 β , IL-6, IL-11, and IL-17. They cause bone loss by reducing osteoprotegerin (OPG) production and stimulating RANKL expression in osteoblasts and stromal cells. The NO inhibition of RAW 264.7 macrophages showed a

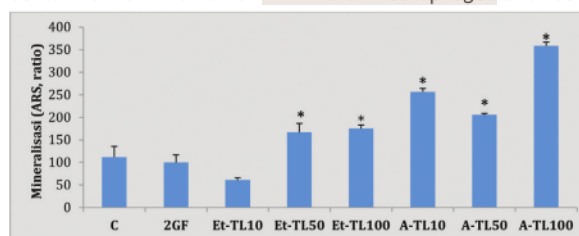


Figure 5. The effects of samples at 10, 50, and 100 µg/mL on quantification mineralization assay during 7 days of incubation; Et-TL was 70% ethanol extract, A-TL was aqueous extract. ARS dye was measured by cetylpyridinium chloride extraction. The results are shown as % with average ± standard deviation. *p<0.05 with respect to 2GF
ARS: Alizarin red staining

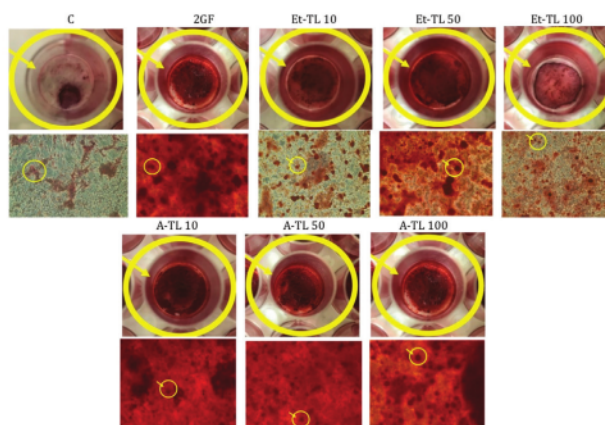


Figure 6. Histochemical staining of mineral deposition of samples at 10, 50, and 100 µg/mL; Et-TL was 70% ethanol extract, A-TL was aqueous extract. They were visualized using ARS staining (100x magnification). Red staining represents mineral deposition
ARS: Alizarin red staining

decrease in cytokine inflammation, thus preventing bone loss by escalating RANKL and OPG levels.²¹

NO inhibition through the p38, JNK, and NF- κ B signaling pathways' retardation is associated with inflammatory response inhibition and osteoclastogenesis suppression. Yeh et al.²¹ showed that Cur liposomes, as samples, inhibited NO production in RAW 264.7 macrophages and prevented osteoclast differentiation by increasing the OPG/RANKL ratio and decreasing cathepsin K regulation and tartrate-resistant acid phosphatase expression. The high NO inhibition and OPG/RANKL ratio lead to a reduction in osteoclast activation and a rise in the number of osteoblasts, so that the effect of osteoporosis can be reduced.

In the present study, we evaluated the 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 in the control group. The 70% ethanol extract of deer antler showed a higher reaction rate in lowering LPS-stimulated RAW 264.7 than that of aqueous extract (Figure 2). Neither of them had any cytotoxic effect on RAW 264.7 macrophages (Figure 1). According to Choi et al.²⁶ the ability of the methanol fraction from deer bone in reducing NO production was the result of decreased regulation and expression of mRNA from pro-inflammatory agents, such as IL-1 β , IL-12 β , and cyclooxygenase. The methanol fraction containing sugar played an essential part in lowering reactions of inflammatory by managing agents and pro-inflammatory cytokines. Pro-inflammatory cytokine production in large quantities can be found in several conditions, such as arthritis, rheumatoid arthritis, osteoarthritis, osteoporosis, and cancer.^{27,28}

The osteoblast phenotype for bone mineralization is obtained in two phases. In the first phase the mature contents and characteristic protein related to the bone cell phenotype such as ALP are detected and in the second phase the contents are mineralized by calcium deposition. A spongy bone layer is formed around the original cartilage and the space between the spongy bones is loaded with bone content and becomes solid bone.

Deer antler is able to stimulate osteoblast proliferation and protein contents in bone such as collagen type 1 and bone sialoprotein and so the antler can trigger osteoblast differentiation through mineralized nodule formation. As expected, the 70% ethanol and aqueous extracts of deer antler increased ALP activity constantly (Figure 4). The result is similar to that in the research reported by Lee et al.¹⁷ in which cells plated in media with aqueous extract had more ALP activity than the 70% ethanol extract. Deer antler significantly increased osteoblast proliferation until 119% of the basic value, stimulated expression of mRNA and ALP activity in the range of 50-100 μ g/mL, and increased type 1 collagen mRNA expression and mineralization to be more than 183%.¹⁷

Interestingly, ALP has been proven to be an enzyme that plays an essential role in the mineralization process. Human bone abnormalities and hypophosphatasia can occur due to the absence of expression of ALP bone form^{29,30} and described by a

lack of bone mineral deposition (rickets). ALP is important for mineralization but its purpose has not yet been fully clarified. We continued to evaluate the effect of 70% ethanol and aqueous extracts of deer antler on mineralization activity (Figure 5).

The new bone tissue formed will be implicated in the mechanism of osteoblast proliferation, osteoblast differentiation, and mineralization. The 7F2 osteoblast cells started differentiating on day 4. The ALP assay is displayed as an initial term marker of differentiated cells. Then the mineralization of 7F2 osteoblast cells produced by ARS was monitored. Figure 6 shows that the extracts stimulated the mineralization of 7F2 osteoblast cells. The stimulation of aqueous extract at 100 μ g/mL was two times higher than that of 70% ethanol extract. This difference may be as a result of a reduction in deer antler extract toxicity or chemical constituents in both extracts.

CONCLUSION

The 70% ethanol and aqueous extracts of *R. unicolor* antler from Kalimantan had a role in bone remodeling. The aqueous extract stimulated higher bone differentiation and mineralization than the 70% ethanol extract with an ALP value of 65% and twice the ARS value. However, the 70% ethanol extract displayed higher NO inhibition activity than aqueous extract with an inhibition value of 40%. Therefore, the extracts can be considered to effectively degrade inflammatory marker expression in osteoblasts and preserve osteoblast functions.

ACKNOWLEDGMENTS

This research was supported by Airlangga University (No. 886/UN3/2018) and was a collaboration between the Faculty of Pharmacy Airlangga University, UPTD of East Kalimantan, and National Chiayi University Taiwan.

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

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