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Yam (*Dioscorea batatas*) Root and Bark Extracts Stimulate Osteoblast Mineralization by Increasing Ca and P Accumulation and Alkaline Phosphatase Activity

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ABSTRACT: Yam (*Dioscorea batatas*) is widely consumed as functional food for health promotion mainly in East Asia countries. We assessed whether yam root (tuber) or bark (peel) extracts stimulated the activity of osteoblasts for osteogenesis. MC3T3-E1 cells (mouse osteoblasts) were treated with yam root extracts (water or methanol) (study I) or bark extracts (water or hexane) (study II) within 0∼10 μg/mL during the periods of osteoblast proliferation (5∼10 day), matrix maturation (11∼15 day) and mineralization (16∼20 day) as appropriate. In study I, both yam root water and methanol extracts increased cell proliferation as concentration-dependent manner. Cellular collagen synthesis and alkaline phosphatase (ALP) activity, both the indicators of bone matrix protein and inorganic phosphate production for calcification respectively, were also increased by yam root water and methanol extract. Osteoblast calcification as cell matrix Ca and P accumulation was also increased by the addition of yam root extracts. In study II, yam bark extracts (water and hexane) increased osteoblast proliferation and differentiation, as collagen synthesis and ALP activity and osteoblast matrix Ca and P deposition. The study results suggested that both yam root and bark extracts stimulate osteogenic function in osteoblasts by stimulating bone matrix maturation by increasing collagen synthesis, ALP activity, and matrix mineralization.

Keywords: yam root and bark, osteogenesis, collagen, alkaline phosphatase, mineralization

INTRODUCTION

Bone formation is maintained by balance of the action of osteoblasts (bone forming cells) and osteoclasts (bone resorbing cells), by which it would be desirable to keep normal healthy osteoblasts to form bone tissue. The pattern that osteoblasts form bone tissue is characterized by the stage of cell proliferation, differentiation and matrix mineralization (1,2). During osteoblasts differentiation, osteoblasts form adequately spread collagenous extracellular matrix (ECM) with collagen proteins and synthesize osteoblasts-specific marker proteins such as alkaline phosphatase (ALP), osteopontin and osteocalcin. Matrix mineralization begins after a few days of initial induction of osteoblasts marker proteins, along with the calcium (Ca) and phosphate (P) accumulation up to late stage of mineralization (3-5).

Osteoblastic MC3T3-E1 cells are osteoblast precursor cell line which is derived from mouse calvaria (2). MC3T3-E1 cells showed a time-dependent and sequential expression of osteoblast characteristics which are similar to bone formation *in vivo*, therefore this cell line provides useful model for osteogenic activity (6). For this reasons, MC3T3-E1 cells are in use for bone-formation study, such as to assess the osteogenic function of nutrients or phytochemicals (7,8). In this study, we used MC3T3-E1 cells as *in vitro* model for studying osteogenic effect of yam extracts.

Yam (*Dioscorea batatas*) is widely distributed and cultivated in East Asia countries, including Korea, China and Taiwan. Yam was used originally as oriental folk medicine, however, recently it becomes popular to be consumed as healthy food. Yam is considered as good for health, because it contains various phytochemicals. Among them, steroidal saponin (diosgenin) is the most prominent component (9). Yam is considered as having

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anti-obesity function, which is recently confirmed in breast cancer cell model study for ameliorating cancer incidence which can occur in high-fed diet (10). The glycoprotein which was isolated from yam tubers increases immune action by stimulating the induction of inducible nitric oxide syntheses (iNOS) in macrophages (11). The anti-inflammatory activity of yam bark is also reported in mouse bone marrow-derived mast cells (12), and in RAW 264.7 cells (13). The anti-inflammatory activity of yam peel extracts particulary *in vivo* was also confirmed in rat model under carbon tetrachloride-induced hepatotoxicity condition (14). Also, yam showed the anti-diabetic effect which ameliorated insulin resistance in high fat diet-fed mouse model (15).

Diosgenin, as one of major type of saponin in yam components, also showed anti-oxidative and hypolipidemic effects in high-cholesterol fed rat (16). It also showed the function of anti-osteoporosis activity by inducing hypoxia-inducible factor-1 activation, therefore stimulating osteoblasts differentiation (17). Diosgenin compensates bone loss in ovariectomized rat model with the synergistic effent of estrogen, etc (18).

Previously, we reported that diosgenin stimulates osteogenic activity in osteoblasts (MC3T3-E1 cells) by increasing synthesis of bone-marker proteins (collagen and ALP) and protein expression of bone-specific transcription factor, runt-related transcription factor 2 (Runx2) (19). We also reported that yam root extracts (water and ethanol) increased osteoblasts proliferation and synthesis of collagen, a major collageneous protein in bone matrix, in MC3T3-E1 cells (20).

In this study, we tested whether various yam root (study I) and bark extracts (study II) have the potential osteogenic activity by osteoblast function. We tested osteogenic activity by measuring synthesis of major bone matrix proteins, collagen (as bone matrix net-like protein for Ca and P deposition), and ALP (an enzyme producing inorganic phosphate for matrix mineral nucleation) and Ca and P deposition in cell layers.

MATERIALS AND METHODS

Preperation of yam root and bark extracts

Prior to test yam extract effect on osteoblasts, we optimized the choice as the organic solvent for extracting by the measurement of cell viability test (MTT assay) up to 10 days, using various organic solvents. More in details, we tested four different organic solvents [methanol (MeOH), hexane, butanol (BuOH), and H_2O] for yam (*Dioscorea batatas*) roots. For the yam barks (peels), we tested six different solvents [MeOH, hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), BuOH, and H_2O]. Among them, the most optimized extracting solvents were water and methanol for yam root and water and hexane for yam barks on the basic of cell viability of 100±5%. Therefore, in the present studies, we used these selected solvents for the study.

Fresh yam (*D. batatas*) roots and barks washed three times with tap water and cut into small pieces. The small pieces of yam roots and barks were extracted with 99.9% methyl alcohol (Duksan Science, Ansan, Korea) during 12 h at room temperature and this process was repeated three times. Then, yam root and bark extracts were filtered using grade I filter paper (Whatman, Pittsburgh, PA, USA). Yam root (H_2O and MeOH) and bark (H_2O and hexane) filtrates were then evaporated using a rotary evaporator (Tokyo Rikakikai, Tokyo, Japan) at 60°C in water bath. The evaporated samples were then dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) and the stock extracts (60,000 μg/mL) kept on 4°C for further experiments. The osteogenic effect of yam root (study I) and bark (study II) extracts was assessed separately in the present study.

Cell culture

Mouse osteoblastic MC3T3-E1 cells (ATCC CRL-2593) were seeded at a density of 1×10^4 cells/mL and cultured in regular culture of growth media containing α-minimum essential medium (α-MEM, Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco), 100 units/L penicillin and 100 μg/mL streptomycin (Gibco) and 1 mM sodium pyruvate (Sigma) in a humidified atmosphere of 5% CO_2 at 37°C incubator. At 80% confluence, cells were cultured in osteogenic differentiation media (OSM), as growth media supplemented with 50 μg/mL L-ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma). OSM culture was used as normal differentiation control. Cells were treated with 0∼10 μg/mL of yam root extracts (H_2O and MeOH) or yam bark extracts (H_2O and hexane). Vehicle group contains 3 μL DMSO/10 mL (0 μg/mL of yam root and bark extracts) and this condition was applied to all cultures to avoid any experimental error due to the amount of DMSO.

Cell proliferation assay

Cell proliferation was measured using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) assay. MC3T3-E1 cells were cultured in 96-well plate and treated with 0∼10 μg/mL of yam root and bark extracts for 10 days. Cell proliferation was measured at 1, 3, and 10 day. Briefly, MTT solution was added to the cells (ratio of MTT solution versus media was 1:10) and then the cells were incubated at 5% CO_2, 37°C incubator for 3 h. Absorbance of formazan by reduction of MTT, as the indication of mitochondrial dehydrogenase activity in viable cells, was measured using microplate reader at

570 nm (Sunrise Absorbance Reader; Tecan Austria GmbH, Grödig, Austria).

Cell lysis and protein concentration

MC3T3-E1 cells were cultured in 6-well plate and treated with 0～10 μg/mL of yam root and bark extracts for 9 days (at late proliferation period) and 15 days (at osteoblast maturation/differentiation period). After treatment, cells were harvested and lysed with radioimmunoprecipitation assay (RIPA) buffer (GenDEPOT, Houston, TX, USA). Protein concentration was measured using bicinchoninic acid (BCA) assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

Collagen synthesis assay

Cellular (synthesized) and extracellular (secreted) collagen concentration: Collagen in cellular and extracellular (media) were measured, after being treated with 0～10 μg/mL yam root and bark extracts for 9 and 15 days. The concentration of collagen was measured using the Sircol soluble collagen assay. Briefly, each 50 μL of collagen standards and samples were added to 1.5 mL micro-centrifuge tube and then 1 mL Sircol dye reagent added and mixed. After dying to collagen, unbound dye solution was removed by draining the tubes. Then, 0.1 M sodium hydroxide (NaOH) was added to release bound dye and measured the absorbance at 540 nm.

Collagen staining in cell layers: To measure collagen accumulation, cell layers was stained by van Gieson's method. MC3T3-E1 cells were cultured in 48-well plate and treated with 0～10 μg/mL yam root and bark extracts for 18 days. Cells were washed with phosphate buffered saline (PBS, Gibco) twice and fixed with 2% formaldehyde (Sigma) for 10 min at 4°C. Then, the cells were stained with van Gieson's dye solution for 20 min at room temperature and rinsed with deionized water (dH_2O) twice. The cell morphologies were photographed using phase contrast microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany).

Alkaline phosphatase (ALP) activity assay

Cellular and extracellular ALP activity: ALP activity in cellular (in cell lysates, synthesized) and extracellular (in media, secreted) were measured from MC3T3-E1 cells treated with 0～10 μg/mL yam root (H_2O and MeOH) and yam bark (H_2O and hexane) extracts. The ALP activity was measured at 9 and 15 days by spectrometric method using 4-nitrophenyl phosphate di(2-amino-2-ethyl-1,3-propanediol) salt (*para*-nitrophenyl phosphate; pNPP, Sigma) as ALP substrate. The products of enzyme activity, 4-nitrophenol (*para*-nitrophenol; PNP, Sigma) was measured absorbance at 405 nm for ALP activity as previously described (21). One unit of enzyme activity was defined as producing 1 μmol of PNP per min. The enzyme activity is expressed as 10^{-3} U/mg protein (for cellular ALP activity) and 10^{-3} U/mL medium (for extracellular ALP activity).

ALP activity staining in cell layers: MC3T3-E1 cells were cultured in 48-well plate and treated with 0～10 μg/mL yam root and bark extracts for 15 days. Cell layers were rinsed with PBS and fixed in 2% formaldehyde. Then naphthol AS-BI phosphate (Sigma) as ALP substrate, which is hydrolysed by ALP in cell layers, was added to the cell layers. After the substrate being hydrolyzed, then phosphate and arynaphtholamide were formed. Once, fast red salt dye (Sigma) was added, and then ary-naphtholamide was combined with this dye and measured for ALP activity. Cells were incubated for 30 min or until red color appeared for enzyme activity at 37°C. The cell morphologies were photographed using phase contrast microscope.

Extracellular mineral deposit staining

Alizarin red staining (Ca deposition): MC3T3-E1 cells were cultured in 48-well plate and treated with 0～10 μg/mL of yam root and bark extracts for 18 days. Ca deposition in ECM is the sign of osteoblasts mineralization. Cell layers were stained using Alizarin red S dye which combines with Ca. Cells were washed with PBS twice and then fixed with 2% formaldehyde for 15 min at 4°C. Then, cells were stained with 1% Alizarin red S solution at pH 4.2 for 30 min at room temperature and rinsed with H_2O twice. Ca deposition was shown as red stain and cell morphologies were photographed using phase contrast microscope.

von Kossa staining (P deposition): Cell culture treatment with yam extracts is the same as in Alizarin red staining. Since phosphate ions (PO_4^{2-}) precipitates with Ca ions to synthesize hydroxyapatite [$Ca_5(PO_4)_3(OH)$] as form of bone mineralization, phosphate ion accumulation in ECM was also assessed using von Kossa staining where silver nitrate combines with PO_4^{2-}. After fixation of cells with 2% formaldehyde for 15 min at 4°C, cells were incubated with 3% silver nitrate at room temperature under ultraviolet light for 5 min. Phosphate accumulation was shown as dark brown stain and cell morphologies were photographed using phase contrast microscope.

Statistical analysis

Data were analyzed using software SPSS 21 (IBM, Rochester, MN, USA). Values for cell proliferation, protein and collagen concentration and ALP activity are presented as mean±SEM (n=6). The data analysis was performed using one way analysis of variance (ANOVA) and Tukey's HSD test ($P<0.05$), if any significance among means were observed.

RESULTS

Study I. Osteogenic effect of yam root extracts (by H_2O and MeOH) in MC3T3-E1 cells

Yam root extracts (H_2O and MeOH) stimulated osteoblasts proliferation and cellular protein synthesis: Yam root extracts by H_2O (Fig. 1A) and MeOH (Fig. 1B) increased osteoblasts (MC3T3-E1 cells) proliferation at 5 and 10 day at the concentration of 10 μg/mL, compare to normal OSM control. Cellular protein synthesis was also increased as the addition of yam root extracts increased (mainly within the range of 3~10 μg/mL) in both H_2O (Fig. 1C) and MeOH (Fig. 1D) extracts. There was no significance between vehicle (0 μg of yam extract/mL, DMSO only) and normal OSM, which means no DMSO as solvent effect was accounted.

Yam root extracts (H_2O and MeOH) increased cellular osteogenic proteins (collagen and ALP) synthesis and activity: Collagen is the form of major collagenous protein in ECM with the function of net-like protein for holding Ca or P ions for matrix calcification. Yam root H_2O (Fig. 2A) and MeOH (Fig. 2B) extracts stimulated cellular collagen synthesis with concentration-dependent manner (Fig. 2A and 2B, upper panels). The collagen accumulation in osteoblast layers as shown in pink stains was also increased as the addition of yam root extracts increased (the images below of Fig. 2A and 2B).

ALP produces inorganic phosphate from phosphate compounds and provides inorganic phosphate for matrix mineralization as the form of hydroxyapatite (phosphate compound). ALP activity was also increased as the addition of yam root extracts (Fig. 2C and 2D) increased. The stimulated function of ALP activity by yam root extracts was also confirmed by staining of products of ALP enzyme activity in osteoblasts (the images below Fig. 2C and 2D).

Yam root extracts (H_2O and MeOH) increased Ca and P deposition in osteoblast layers: As the addition of yam root extracts (Fig. 3A, upper and lower images) increased, Ca deposition (in dark spots) was increased more in osteoblast layers. P accumulation by H_2O and MeOH extracts was also increased P ion (in dark grey) deposition in osteoblast layers (Fig. 3B, upper and lower images).

Study II. Osteogenic effect of yam bark extracts (by H_2O and hexane) in MC3T3-E1 cells

Yam bark extracts (H_2O and hexane) stimulated cell proliferation and protein concentration in osteoblasts: Yam bark extracts (by H_2O and hexane) stimulated osteoblasts proliferation, especially at culture day 5 and 10 (osteoblasts

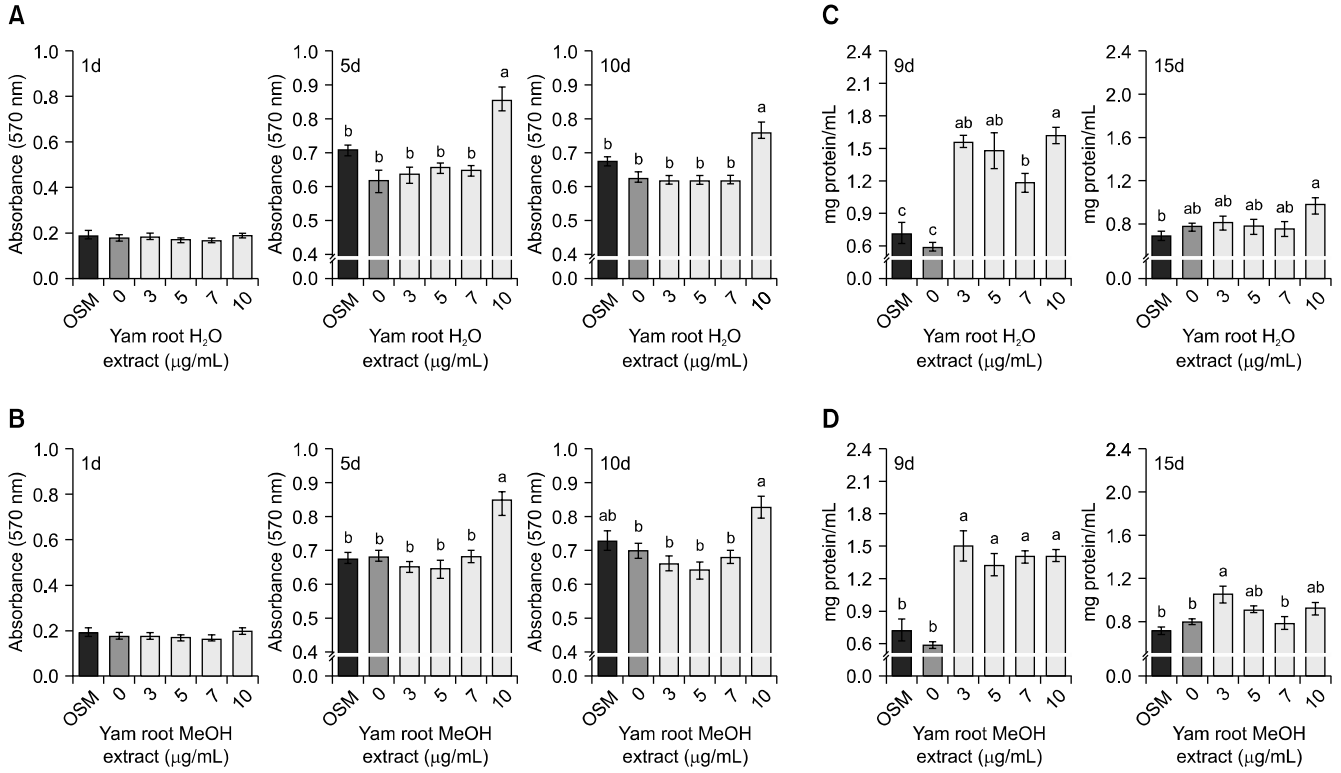

Fig. 1. Yam root extracts (H_2O and MeOH) increased cell proliferation (A, B) and protein concentration (C, D) in osteoblasts. Both H_2O (A) and MeOH (B) extract stimulated osteoblasts proliferation up to 10 days. Protein concentration was also increased concentration-dependent manner by both H_2O or MeOH yam root extracts (C, D). Cell proliferation was presented as the absorbance of formazan which is the indication of products of viable cell by MTT assay. Cellular protein concentration was measured by BCA assay. MC3T3-E1 cells were cultured with 0~10 μg/mL yam extracts (H_2O or MeOH) up to 15 days. Values were analyzed by one-way ANOVA, Tukey test for mean comparison as *post hoc*. Different letters (a-c) mean significant difference among the groups. Mean±SEM (n=6). OSM: normal osteogenic differentiation medium.

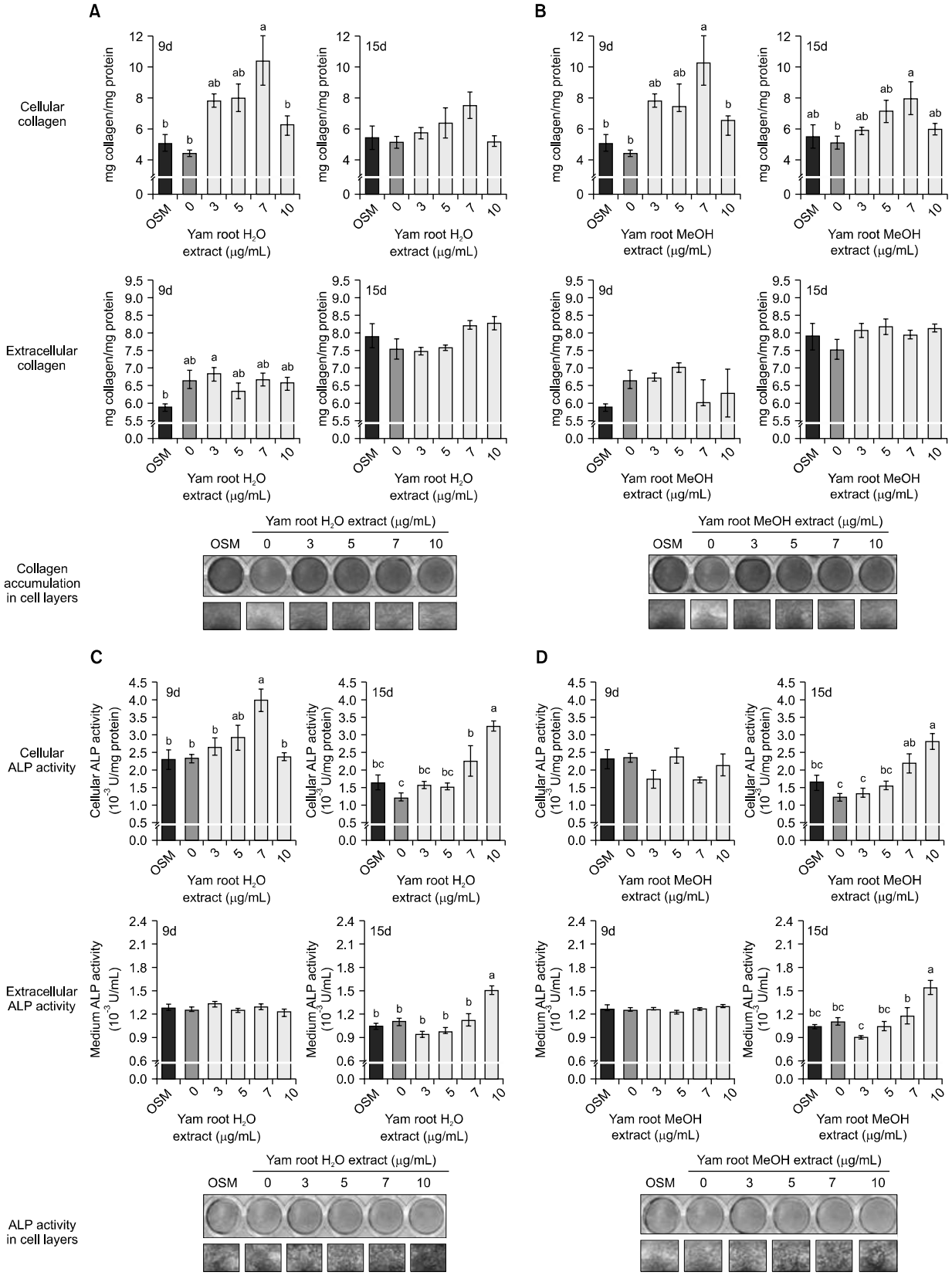

Fig. 2. Yam root extracts (H_2O and MeOH) increased extracellular matrix proteins (collagen and ALP) synthesis and enzyme activity in osteoblasts, respectively. Cellular (synthesized) collagen concentration increased, both yam root H_2O (A) and MeOH (B) extracts being added more. Collagen accumulation in cell layers increased with the extracts concentration-dependent manner (the image below A and B, shown as dark sopts). ALP activity in both cellular and extracellular condition increased by yam extracts (C for H_2O and D for MeOH) at day 15, which was consistent with staining of ALP activity in cell layers (shown as dark spots in the images below C and D). The experimental condition was the same in Fig. 1.

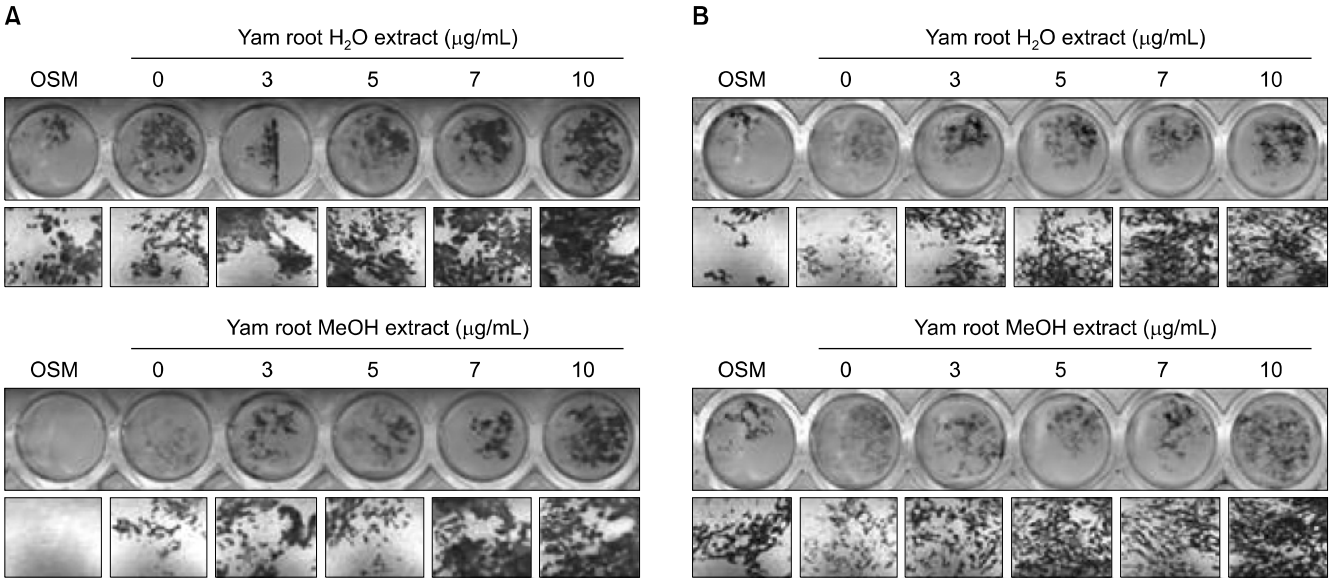

Fig. 3. Yam root extracts (H_2O and MeOH) increased Ca (A) and P (B) accumulation in osteoblast layers. MC3T3-E1 cells were cultured with 0~10 μg/mL yam root H_2O or MeOH extracts up to 18 days (osteoblast mineralization period). Ca ion was stained by Alizarin red and P (as phosphate ion) deposition by von Kossa. OSM: normal osteogenic differentiation medium.

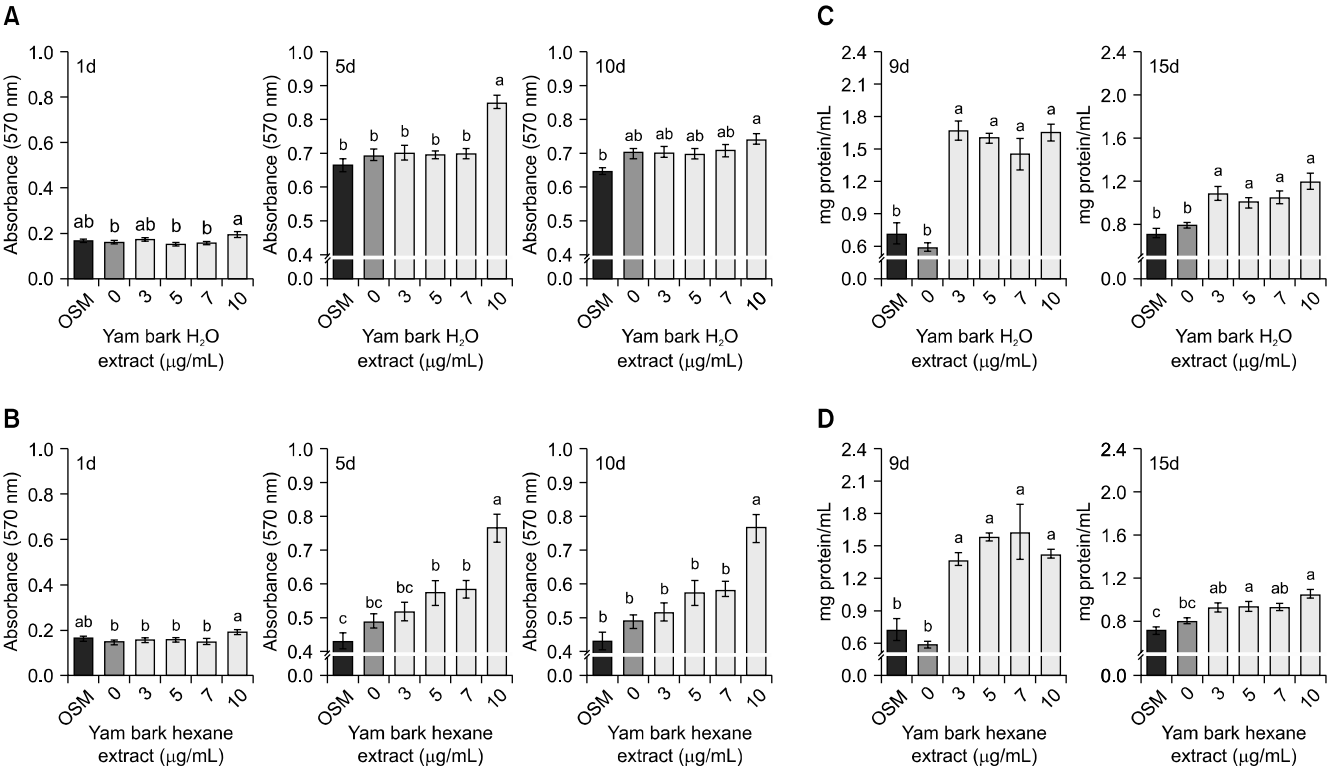

Fig. 4. Yam bark extracts (H_2O and hexane) stimulated cell proliferation (A, B) and protein concentration (C, D) in osteoblasts. Cellular protein concentration (expressed as mg protein/mL cell lysates) was also increased with yam bark extracts concentration-dependent manner. Cell proliferation was measured by MTT assay and protein concentration by BCA assay. MC3T3-E1 cells were cultured with 0~10 μg/mL yam bark extracts (H_2O or hexane) up to 15 days. Different letters (a-c) mean significant difference among the groups. Mean±SEM (n=6). OSM: normal osteogenic differentiation medium.

proliferation and maturation period) (Fig. 4A and 4B). The protein concentration also prominently increased by the addition of bark extracts, especially between 3~10 μg/mL in both H_2O (Fig. 4C) and hexane (Fig. 4D) at day 9.

Yam bark extracts (H_2O and hexane) increased the synthesis and activity of matrix proteins (collagen and ALP) in osteoblasts: Collagen synthesis (cellular) increased as the addition of yam bark extracts (by H_2O and hexane) increased, especially within the range of 3~10 μg/mL (Fig. 5A and 5B).

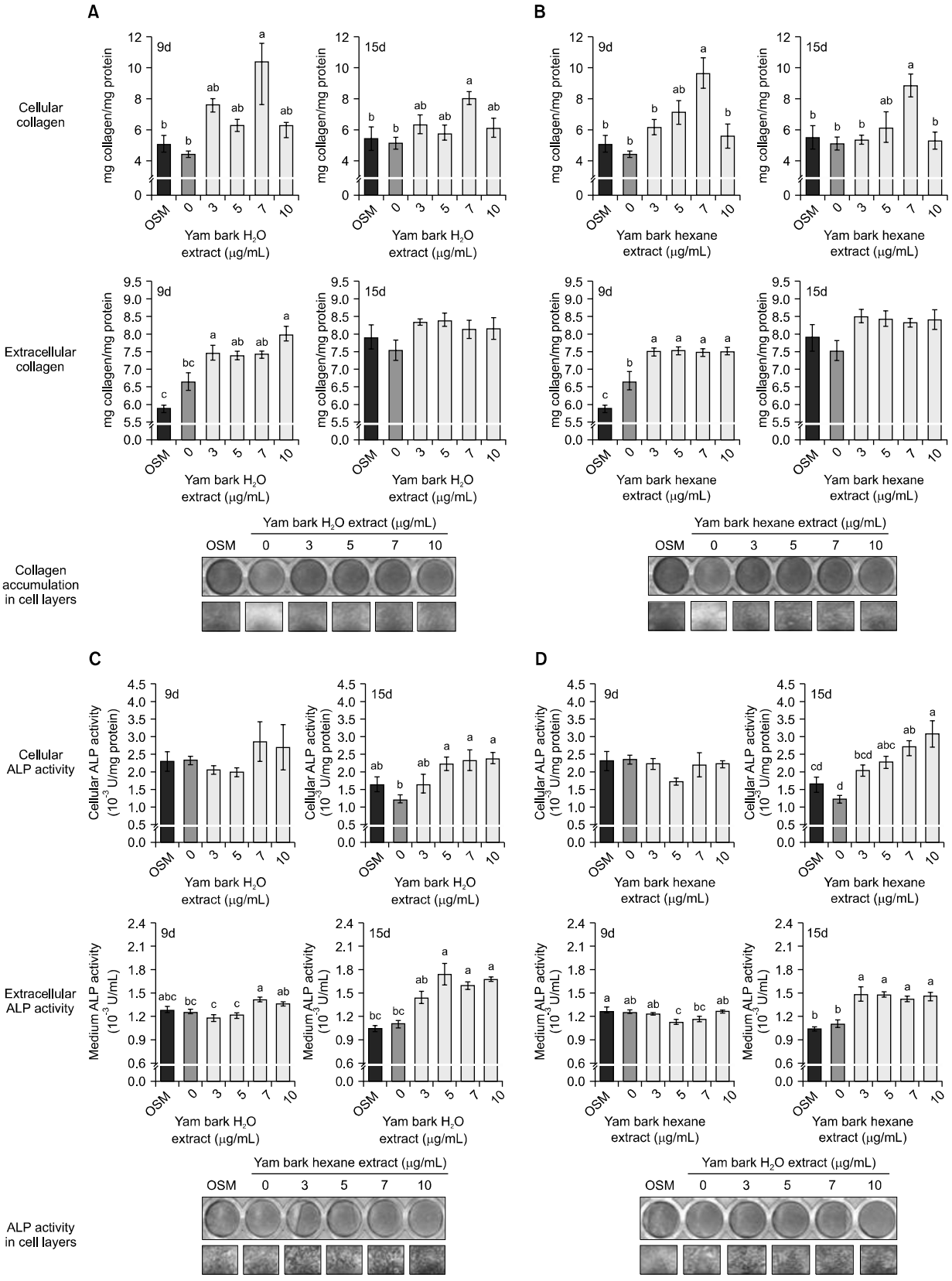

Fig. 5. Yam bark extracts (H_2O and hexane) stimulated collagen synthesis (A, B) and ALP activity (C, D) in osteoblasts. Cellular (synthesized) collagen synthesis and accumulation in cell layers (the images below A and B) increased when the addition of yam bark extract increased in both H_2O (A) and hexane (B) extracts. ALP activity in cellular (synthesized) and extracellular (secreted) also increased as the addition of yam bark H_2O (C) and hexane (D) extracts increased. MC3T3-E1 cells were cultured with yam bark H_2O and hexane extracts (0~10 μg/mL) up to 15 days and collagen concentration and ALP activity measured. Values were analyzed by Tukey as *post hoc*, one-way ANOVA. Different letters (a-c) mean significant difference among the groups. Mean±SEM (n=6). OSM: normal osteogenic differentiation medium.

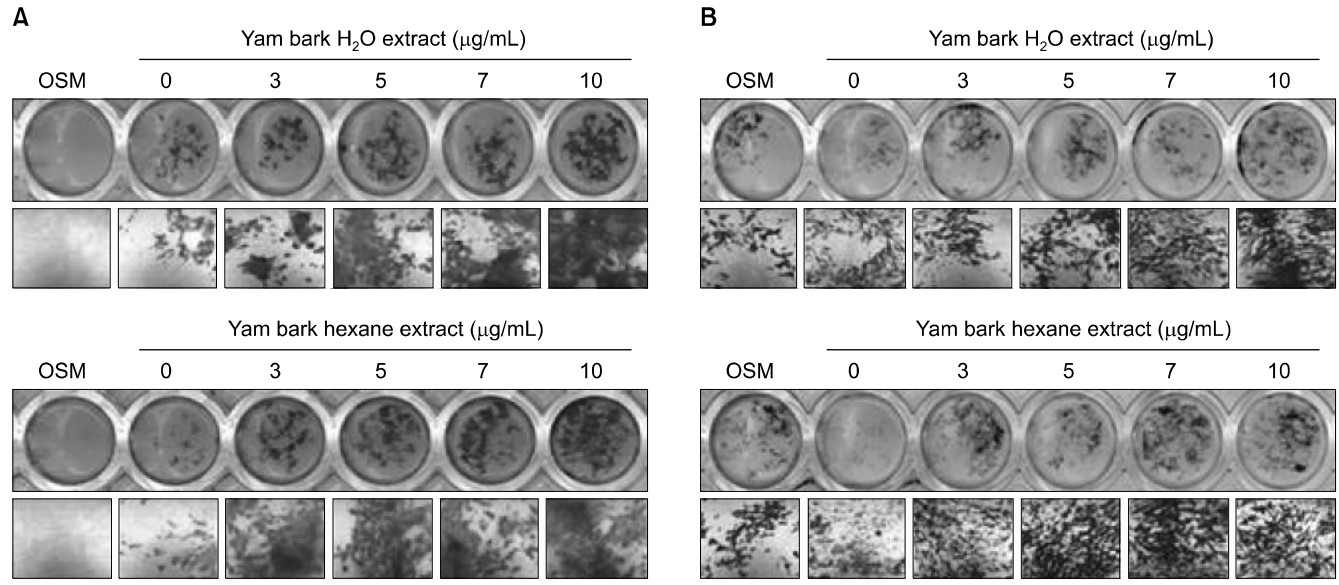

Fig. 6. Yam bark extracts (H_2O and hexane) increased Ca (A) and P (B) deposition in osteoblasts. Ca and P (as phosphate ion) are shown as dark spots. MC3T3-E1 cells were cultured with 0~10 μg/mL yam bark extracts (H_2O and hexane) up to 18 days. OSM: normal osteogenic differentiation medium.

ALP activity increased as concentration-dependent pattern of yam bark extracts (by H_2O and hexane, Fig 5C and 5D), especially at culture day 15.

Yam bark extracts (H_2O and hexane) increased matrix Ca and P deposition in osteoblast layers: Yam bark extracts (H_2O and hexane) increased Ca and P deposition (Fig. 6A and 6B) in osteoblast layers with concentration-dependent manner at culture day of 18 (osteoblasts mineralization period).

DISCUSSION

In the present study, we assessed whether yam bark (by H_2O and hexane) as well as yam root extracts (by H_2O and MeOH) have the osteogenic function *in vitro* model, using mouse osteoblastic MC3T3-E1 cells. The study results showed that both yam root and bark extracts showed stimulatory osteogenic function, mainly 1) by stimulating osteoblasts differentiation by increasing synthesis of collagen (a collagenous matrix protein for ECM in bone tissue) and bone related enzyme ALP (a enzyme producing inorganic phosphate as the nuclear of hydroxyapatite in matrix calcification) and 2) by increasing matrix calcification through increasing accumulation of Ca and P deposition in osteoblast layers. To our limited knowledge, the positive potential of yam bark (by H_2O and hexane) for osteogenesis by osteoblast would be the first to be reported.

In our previous study that yam root ethanol extract showed the potential for osteogenesis by stimulating osteoblasts proliferation and collagen synthesis (20). We reported in this study the positive effect for osteogenesis by yam root (by H_2O and MeOH). In the present study, we also showed that the extracts of yam barks (peels) (by H_2O and hexane) have osteogenic potential by stimulating osteoblasts differentiation (collagen synthesis and ALP activity) and osteoblasts matrix calcification by Ca and P accumulation.

Osteoblasts are the cells to rebuild lost bone and they are derived from local stem cells within the mesenchyme (1). Osteoblasts proceed the phases of bone formation as early proliferation, matrix maturation (with synthesis and accumulation of matrix proteins, including collagen and ALP), and finally mineralization as Ca and P deposition (1). We chose MC3T3-E1 cells as osteoblasts model, since this cells produce osteogenic markers (such as collagen and ALP) and process events (osteoblasts differentiation and mineralization) for bone formation. In the present study, yam bark as well as root extracts increased osteoblasts proliferation up to day 10 (proliferation period) (Fig. 1 and 4). The stimulatory effect for osteoblasts proliferation is more prominent by yam bark extracts from early stage of proliferation at day 1. Osteoblasts proliferation is essential for osteoblasts differentiation, because it is immediately followed for bone tissue formation (22).

Collagen is the most abundant ECM protein in bone tissue. Collagen is synthesized and secreted outside osteoblasts and composes structural support fixture for osteoblast differentiation in bone formation. More in details, collagen composes collagenous net-like structure in bone matrix, where inorganic phosphates (which are produced by enzyme, ALP) and Ca ions are entrapped for bone mineralization (23,24). Therefore, collagen synthesis and ALP function are widely recognized as

osteoblastic activity (25,26). In the present study, yam bark (H_2O and hexane) as well as yam root (H_2O and MeOH) extracts increased collagen synthesis and its accumulation in osteoblast matrix (Fig. 2A, 2B and 5A, 5B). Also, ALP activity was increased by yam bark extracts as well as yam root extracts (Fig. 2C, 2D and 5C, 5D). This means that both yam root and bark extracts has the stimulatory effect for osteoblasts differentiation and matrix maturation by stimulating collagen synthesis and ALP activity.

Osteoblasts mineralization is advanced stage of bone formation after bone matrix and cellular maturation of collagen synthesis and ALP activity period (27). Bone mineralization requires an elevated level of Ca and P ion products, which is accomplished either by adding organic phosphates such as β-glycerophosphate (28). After osteoblasts maturation period, mineralization is followed by deposition of Ca and P ions on matrix. In ECM, hydroxyapatite as form of phosphate-containing compound is composed for bone calcification. In the present study, Ca and P accumulation in osteoblast layers increased, as the addition of yam root and bark extracts increased (Fig. 3 and 6).

While the beneficial effects of yam root (tuber) are reported in various physiological benefits, the physiological effect of bark (peel) of yam has limited. In this study, we reported that yam bark has also stimulatory function for osteogenensis by stimulating osteoblasts proliferation and differentiation by increasing collagen synthesis and ALP activity as well as Ca and P deposition. In conclusion, we suggest that yam bark (H_2O and hexane) extracts as well as yam root (H_2O and MeOH) extracts has the beneficial effect for the osteogenesis in osteoblasts by stimulating osteoblast differentiation and cell matrix calcification. Further studies are needed for clarifying how and whereby the yam bark extracts and components stimulate osteoblast bone formation.

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AUTHOR DISCLOSURE STATEMENT

The authors declared no conflict of interests.

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