IN VITRO BONE FORMATION INDUCING ACTIVITY OF SELECTED THAI TRADITIONAL FORMULATIONS FOR THE PROMOTION OF WOMEN'S HEALTH

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ABSTRACT: In Thai traditional medicine, there are a number of formulations for the promotion of women's health. We investigated the effects of the aqueous and ethanolic extracts of two selected multiple-plant formulations, i.e., blood tonic for women's health (YHA1 and YHA2), on bone formation by assessing the effects of these formulations on the proliferation, differentiation and mineralization of MC3T3-E1 osteoblast-like cells. The aqueous and ethanolic extracts of the two selected multiple-plant formulations at a concentration of 7.8 μg/ml significantly stimulated cell proliferation. This study is the first to demonstrate the positive bone formation effects of these Thai traditional formulations, which will be useful for further *in vivo* and in clinical studies.

Keywords: Bone formation activity, MC3T3-E1 osteoblast-like cells, Phytoestrogens, Postmenopausal women, Thai traditional medicine

INTRODUCTION

Osteoporosis is a major public health concern. It is a metabolic bone disease that is characterized by low bone mass and micro-architectural deterioration of bone tissue. This disease increases the risk of bone fragility and fracture, which increase morbidity and mortality in aging postmenopausal women [1, 2].

Estrogen deficiency in postmenopausal women is a cause of rapid bone loss after menopause [3]. Hormone replacement therapy is a standard treatment that is currently used to prevent bone loss. However, the adverse effects of this therapy on reproductive organs, such as cancers of the breast and uterus, are of great concern with long-term treatment. Natural products, such as phytoestrogens, have been considered as complementary and alternative medicines [4]. These natural products have beneficial anti-osteoporotic effects by stimulating bone formation but have less severe adverse effects on reproductive organs [5, 6].

In Thai traditional medicine, there are herbal medicines used specifically to promote women's health and to relieve female-specific symptoms, i.e., premenstrual, postpartum, menopausal, and postmenopausal symptoms [7]. Blood tonic formulation for women's health or Yha-Bam-Rung-Lo-Hit-Sa-Tri (YHA) is a group of Thai traditional formulations used to promote women's health and to treat menstrual disorders. Modern medical science has demonstrated that estrogen involved in menstrual disorders and hormone-related diseases, including osteoporosis. Therefore, YHA, which is used for the treatment of hormone-related symptoms, might have beneficial effects on osteoporosis.

Two multiple-plant formulations were selected for investigation of their anti-osteoporotic activities. Furthermore, some of the plants in these formulations have been reported to have estrogenic activities. The first formulation, YHA1, was

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Table 1 Compositions of YHA1 and YHA2

Formula	Compositions
YHA1	*Angelica dahurica* Benth. root, *Cinnamomum verum* J.Presl stem bark, *Cinnamomum* sp. leaf, *Mesua ferrea* L. flower, *Myristica fragrans* Houtt aril, *Myristica fragrans* Houtt. endosperm, *Nigella sativa* L. seed, *Piper retrofractum* Vahl infruitescence, *Piper nigrum* L. fruit, *Zingiber officinale* Roscoe rhizome, *Syzygium aromaticum* (L.) Merr. & L.M.Perry flower bud
YHA2	*Alyxia reinwardtii* Blume bark, *Amomum testaceum* Ridl. fruit, *Anaxagorea* sp. bark, *Anethum graveolens* L. fruit, *Angelica dahurica* Benth. root, *Angelica sinensis* (Oliv.) Diels root, *Aquilaria crassna* Pierre ex Lecomte heartwood, *Arcangelisia flava* (L.) Merr. stem, *Artemisia annua* L. arial parts, *Atractylodes lancea* (Thung.) DC. rhizome, *Avicennia marina* Forssk. heartwood, *Bixa orellana* L. flower, *Caesalpinia sappan* L. heartwood, *Cinnamomum verum* J.Presl inner stem bark, *Cuminum cyminum* L. fruit, *Dracaena cochinchinensis* Hort. ex Baker heartwood, *Foeniculum vulgar*e Mill. fruit, *Laccifera chinensis* Mahdihassan resinous secretion, *Lepidium sativum* L. seed, *Ligusticum chuanxiong* Hort rhizome, *Mammea siamensis* Kosterm. flower, *Mesua ferrea* L. flower, *Mimusops elengi* L. flower, *Myristica fragrans* Houtt. aril, *Myristica fragrans* Houtt. endosperm, *Nelumbo nucifera* Gaertn. stamen, *Nigella sativa* L. seed, *Piper retrofractum* Vahl infruitescence, *Piper* sp. stem, *Piper sarmentosum* Roxb. whole plant, *Plumbago indica* L. root, *Senna garrettiana* (Craib) Irwin & Barneby heartwood, *Syzygium aromaticum* (L.) Merr. & L.M.Perry flower bud, *Terminalia bellirica* (Gaertn.) Roxb. fruit, *Terminalia chebula* Retz. fruit, *Terminalia citrina* (Gaertn.) Roxb. ex Fleming fruit, *Urceola rosea* (Hook. & Arn.) D.J.Middleton stem, *Zingiber officinale* Roscoe rhizome

recorded in Phathayasastra sangkhraha, the Thai traditional medicine textbook. This formulation composes of 10 medicinal plants [7], three of them have been reported to possess estrogenic activity *(Angelica dahurica* Benth. [8], *Nigella sativa* L. [9], *Zingiber officinale* Roscoe [10, 11]). The second formulation, YHA2, was recorded in a list of Thai traditional household remedies. It composes of 36 medicinal plants and one animal product [12]. Five medicinal plants contained in YHA2 *(A. dahurica* Benth. [8], *A. sinensis* (Oliv.) Diels [13-15], *N. sativa* L. [9], *Plumbago indica* L. [16] and *Z. officinale* Roscoe [10, 11] have been reported to possess estrogenic activity.

Osteoporosis relates to accelerating bone remodeling due to an imbalance between bone resorption regulated by osteoclasts and bone formation regulated by osteoblasts [3, 17]. Bone formation activity was selected as an indicator of anti-osteoporotic activity. The MC3T3-E1 cell line, a preosteoblast cell line, is a well-known model for investigating bone formation activity. This cell has the capacity to differentiate into mature osteoblasts [18] and goes through three normal developmental stages: cell proliferation, differentiation and matrix mineralization [19]. Thus, this cell line was selected as a model for investigating bone formation activity.

Thus, the bone formation activities of the two selected Thai traditional formulations, YHA1, and YHA2, were investigated using MC3T3-E1 osteoblast-like cells to support the use of Thai traditional medicines to promote women's health.

MATERIALS AND METHODS

Plant materials

All materials contained in YHA1 and YHA2 (Table 1) were purchased from a Thai traditional drugstore in Bangkok. The voucher specimens (Collector number: PJ01-PJ40) were deposited in the Mahidol University Herbarium (PBM), Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University. TLC fingerprints of all extracts were performed as reference of the experiment (Figure 1).

Preparation of extracts

All materials were dried at 60-70 °C. The dried ingredients of the multiple-plant formulations were mixed in the proportions recommended for traditional use. After drying and mixing, the dried materials were pulverized and sifted through a no. 40 sieve to give powdered samples.

For extract preparation, the multiple-plant formulations were prepared by aqueous decoction and ethanolic maceration according to the traditional processes. In total, four extracts were prepared, i.e. aqueous extracts and ethanolic extracts of the two multiple-plant formulations (YHA1 and YHA2). For the aqueous extracts, 200 g of dried powdered sample was boiled in distilled water (1:3 w/v) for 30 minutes. The extraction was repeated twice, and the extracts were combined and filtered through filter paper (Whatman no. 1). The filtrate was then lyophilized. The yields of aqueous extracts for YHA1 and YHA2 were 1.09% and 2.18%, respectively. For the ethanolic extracts, 100 g of dried powder was macerated with 80% ethanol

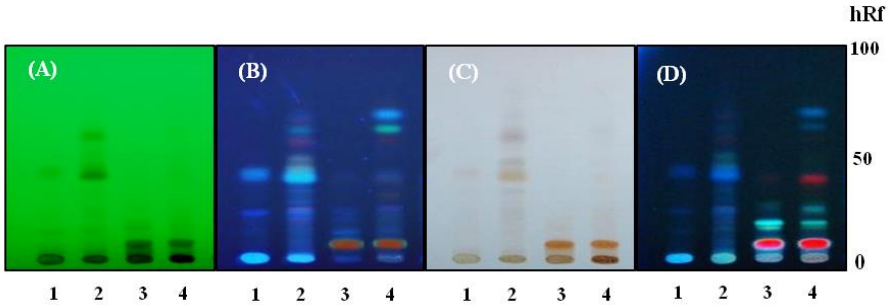

Figure 1 TLC chromatograms of four extracts of selected Thai traditional formulations: (1) aqueous extract of YHA1, (2) ethanolic extract of YHA1, (3) aqueous extract of YHA2, and (4) ethanolic extract of YHA2. The samples were run on TLC silica gel plate (GF254) using the solvent system of toluene:ethyl acetate:acetone:formic acid (20:4:2:1). The chromatograms were detected under (A) UV 254 nm, (B) UV 365 nm, (C) white light after spraying with anisaldehyde/sulfuric acid spray reagent and heated at 120 °C for 5 min, and (D) UV 365 nm after spraying with natural product spray reagent.

at room temperature for 24 hours. The extraction was repeated three times. The extracts were filtered through filter paper, combined and then concentrated under reduced pressure using a rotary evaporator. The yields of the ethanolic extracts of YHA1 and YHA2 were 7.46% and 10.89%, respectively.

The four extracts were prepared as stock solutions at a concentration of 2 mg/ml. The aqueous extracts were diluted with sterile distilled water and the ethanolic extracts were diluted with DMSO. For all experiments, the stock solutions were two-fold serially diluted with culture medium to final concentrations of 7.8-1000 µg/ml. The final concentration of DMSO in the culture medium was 0.5%.

Cell culture

MC3T3-E1 cells subclone 4 (ATCC, Manassas, VA, USA) were maintained in basal medium (BM). The BM was composed of alpha minimum essential medium (α-MEM, GIBCO®, NY, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone®), 1.1 mg/mL $NaHCO_3$, 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of amphotericin B (GIBCO®, NY, USA). The cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell cytotoxicity assay

Cells were seeded in 96-well plates at a density of $5x10^3$ cells/well and cultured for 24 hours to allow cell attachment before the cytotoxicity assay. The cells were treated with various concentrations (7.8-1000 µg/ml) of the extracts. Cells which were not treated with the extracts were served as controls. After incubation for 1, 2, and 3 days, the cytotoxic activity was detected using the MTT assay [20]. Each concentration was tested in six wells, and the experiment was performed three times. The percentage of viable cells was calculated as follows:

$$\% \text{ Cell viability} = \left(\frac{\text{OD sample-OD control}}{\text{OD control}}\right) \times 100$$

The concentration that gave a cell viability less than 70% was defined as cytotoxic [20].

Cell proliferation assay

The cells were seeded at a density of 2 X 10^3 cells/well and cultivated for 24 hours to allow cell attachment. Then, the cells were incubated with or without the extracts (7.8 µg/ml). The cells were harvested after 1, 3, 5 and 7 days of incubation. The effects on cell proliferation were determined using the MTT assay. Each treatment was performed in five wells, and the experiment was performed three times.

Cell differentiation and mineralization

The cells were seeded in 24-well plates at a cell density of $5x10^4$ cells/well and cultivated for 24 hours. Then, the medium was replaced with osteogenic medium (OM) (BM supplemented with 2 mM β-glycerophosphate, 50 µg/ml ascorbic acid and 10^{-8} M dexamethasone) with or without extracts (7.8 µg/ml). The cells were harvested on days 7, 14 and 21 for analysis of cell differentiation and on day 28 for analysis of mineralization.

The effect on differentiation was determined based on the alkaline phosphatase (ALP) activity [18] by the enzymatic conversion of *p*-nitrophenyl phosphate into a yellow product, *p*-nitrophenol (*p*-NP). The *p*-NP concentration was spectrophotometrically measured at 405 nm after 30 min. The enzymatic activity was normalized to the total protein concentration using bovine serum albumin (CB-protein assay™, St. Louis, MO.,

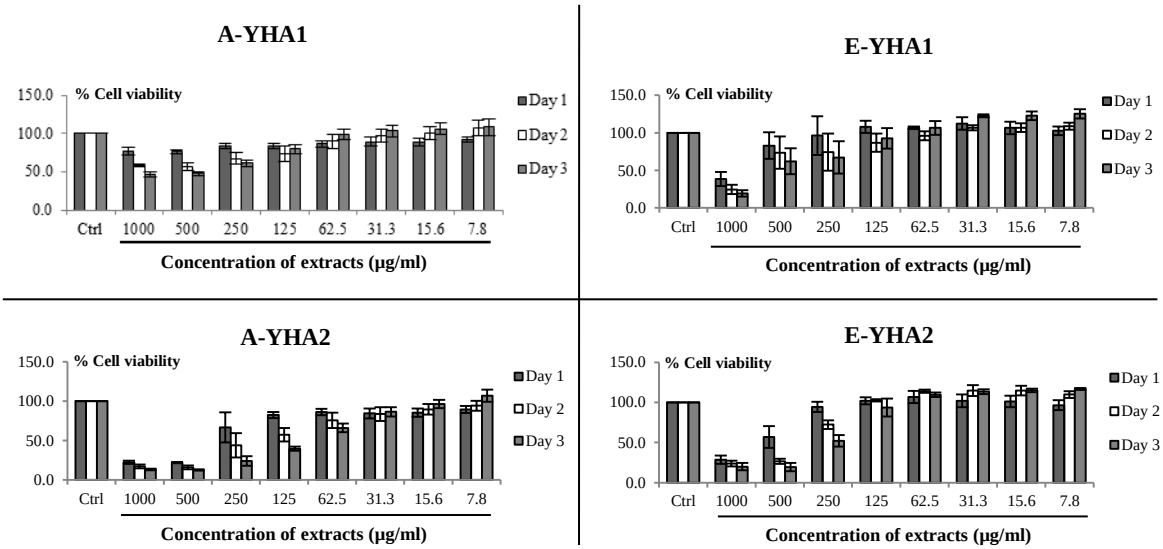

Figure 2 Cytotoxicity effects of four extracts of selected multiple-plant formulations at concentration of 7.8-1000 μg/ml on MC3T3-E1 cells. Cells were detected at day 1, 2, and 3 after incubation. The results were expressed as % cell viability. The extracts that gave cell viability more than 70% when compared with control indicated non-cytotoxicity of cells. Ctrl: control group; A-YHA1: aqueous extract of YHA1; E-YHA1: ethanolic extract of YHA1; A-YHA2: aqueous extract of YHA2; E-YHA2: ethanolic extract of YHA2. Results are expressed as mean ± SEM.

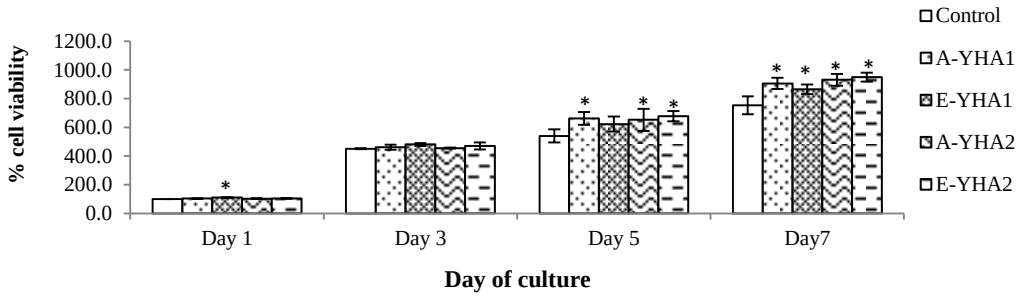

Figure 3 Effect on cell proliferation of MC3T3-E1 cells of four extracts of selected multiple-plant formulations at the concentration of 7.8 μg/ml. The extracts were tested at concentration of 7.8 μg/ml. The percentage of cell viability of the control group at day 1 was set to 100%. Control: basal medium (BM); A-YHA1: aqueous extract of YHA1; E-YHA1: ethanolic extract of YHA1; A-YHA2: aqueous extract of YHA2; E-YHA2: ethanolic extract of YHA2. Results are expressed as mean ± SEM. *p<0.05 compared to control group within the same day.

USA.). ALP activity was reported in units of μM *p*-NP/mg protein/min. For the mineralization analysis, the cells were fixed with ice-cold methanol and stained using 1% Alizarin Red S to determine the amount of calcium deposition. The orange-red calcium nodules were observed under inverted microscope. Each treatment was performed in duplicate wells, and the experiment was performed three times.

Statistical analysis

The results are presented as the mean ± standard error (SEM). The data were analyzed using one-way ANOVA followed by Dunnett's test using SPSS 17.0. The differences between means were considered statistically significant when $p < 0.05$.

RESULTS

Cell cytotoxicity assay

Eight concentrations of the four extracts were analyzed. The aqueous and ethanolic extracts of YHA1, and the ethanolic extract of YHA2 at concentrations lower than 125 μg/ml, and the aqueous extract of YHA2 at concentrations lower than 15.6 μg/ml exhibited no cytotoxic effects (Figure 2). The concentrations of all extracts that did not show 3-day cytotoxicity in MC3T3-E1 cells were in the range of 7.8-15.6 μg/ml. Therefore, the concentration of 7.8 μg/ml was selected to investigate the effects of these extracts on bone formation.

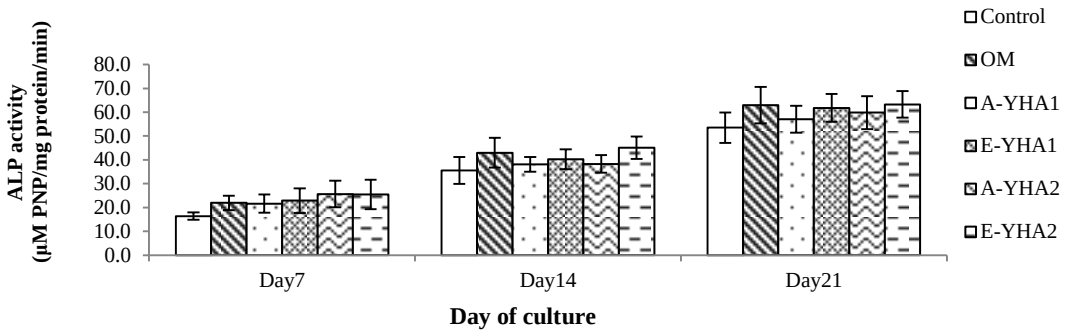

Figure 4 Effect of four extracts of selected multiple-plant formulations at the concentration of 7.8 µg/ml on the differentiation of MC3T3-E1 cells. Alkaline phosphatase (ALP) activity was determined on day 7, 14, and 21 of culture. OM showed higher ALP activity on day 7, 14, and 21 than control. However, all the extracts did not show any different ALP activity when compared to OM. Control: basal medium (BM); OM: osteogenic medium; A-YHA1: aqueous extract of YHA1; E-YHA1: ethanolic extract of YHA1; A-YHA2: aqueous extract of YHA2; E-YHA2: ethanolic extract of YHA2. Results are expressed as mean ± SEM.

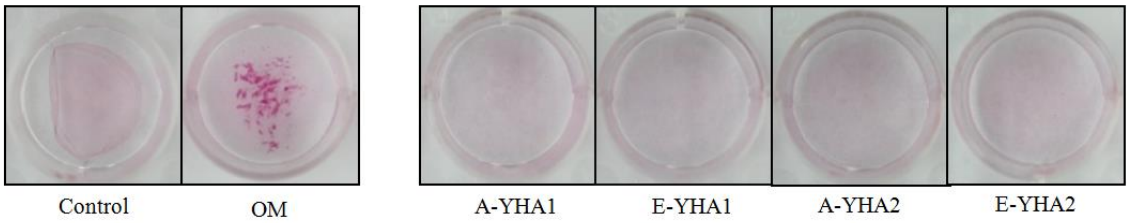

Figure 5 Effect of four extracts of selected multiple-plant formulations at the concentration of 7.8 µg/ml on the mineralization of MC3T3-E1 cells. The cells were cultured in osteogenic media (OM) (α-MEM supplemented with 10% fetal bovine serum, 1.1 mg/mL $NaHCO_3$, 100 units/mL of penicillin, 100 µg/mLof streptomycin, and 0.25 µg/mL of amphotericin B, 10^{-8} M dexamethaxone, 50 µg/ml ascorbic acid, and 2 mM β-glycerophosphate) with or without the extracts at the concentration of 7.8 µg/ml for 28 days. Bone nodules were stained with Alizatin Red S and gave intese red color. OM: osteogenic medium; A-YHA1: aqueous extract of YHA1; E-YHA1: ethanolic extract of YHA1; A-YHA2: aqueous extract of YHA2; E-YHA2: ethanolic extract of YHA2.

Cell proliferation study

The proliferation of MC3T3-E1 cells treated with four extracts (7.8 µg/ml) was observed over a period of 7 days of cultivation (Figure 3). Both the aqueous and ethanolic extracts of the two formulations, YHA1 and YHA2 significantly promoted cell proliferation relative to the control since day 5.

ALP activity

Cell differentiation was observed in all treatments since 7 days after culture and continuously increased (Figure 4). The OM group exhibited a higher level of ALP activity than the control. ALP activity was detected in all extract-treated groups in a time-dependent manner. However, there was no significant difference in the ALP activity when compared to control (OM) which can be implied that the extract did not show any effect on the differentiation process.

Mineralization

Alizarin red S staining is a technique used to detect matrix mineralization. This stain binds with calcium, a composition of hydroxyapatite, and thus all bone nodules show an intense red color. The control (OM) exhibited evidence of new bone nodule formation, which demonstrated the normal mineralization process (Figure 5). Bone nodules were not found in the cells treated with any extracts of the two formulations, YHA1 and YHA2. This result indicated that the extract did not stimulate mineralization of MC3T3-E1 cells.

DISCUSSION

This study suggests that the selected Thai traditional formulations are beneficial to use for promotion of women's health related to bone formation activity. The extracts of two selected Thai traditional formulations (YHA1 and YHA2) induced bone formation by promoting cell proliferation in MC3T3-E1 cells.

The present study demonstrated that the two multiple-plant formulations, YHA1 and YHA2, strongly stimulated the proliferation of MC3T3-E1

cells. This activity might be an effect of some plant compositions. Two medicinal plants among the 36 medicinal plants of YHA2—*Angelica sinensis* (Oliv.) Diels and *Cumimum cyminum* L.—have been reported to stimulate bone formation in human osteoprecursor cells [21] and to increase the bone mechanical strength and calcium content of the tibia in OVX rats [22]. The previous reports also indicated that these plant compositions have anti-osteoporotic activity that was in agreement with our present results for bone formation activity. Thus, the observed effects might be the result of synergistic effect with other plants in the formulation. This activity needs to be further investigated to define the group of active compounds.

This study is the first time demonstrated the positive effect of these selected formulations on bone formation process of MC3T3-E1 cells. The bone formation activity might be related to phytoestrogen presented in the plants which possessed the estrogenic activity. So, the activity of these formulations should be compared with estrogen in further investigation. Moreover, any unidentified compounds can be a candidate. Thus, the active compounds as well as the mechanism of action of these selected formulations should be determined.

In summary, this study reported the stimulating effect on bone formation of the selected Thai traditional formulations, which will be beneficial information for further *in vivo* and clinical investigations and support the use of these medications for the promotion of women's health, especially focusing on osteoporosis.

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