

**OSTEOGENESIS ACTIVITY OF FRACTIONS EXTRACTED FROM
Clinacanthus nutans (Burm. F.) Lindau**

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ABSTRACT

Osteogenesis activity of fractions extracted from *Clinacanthus nutans* was evaluated on an *in vitro* model using osteoblast MC3T3-E1 cells. The results showed that all fractions, including ethanol (EtOH), n-hexane, ethyl acetate (EtOAc) and butanol (BuOH) were not significantly toxic to the osteoblast cells at the test concentrations of 5, 10, 25, and 50 µg/mL. The EtOH and EtOAc fractions exhibited the highest osteogenesis activity in terms of enhancement of alkaline phosphatase (ALP) and mineralization activity of MC3T3-E1 cells, especially the EtOAc fraction which increased ALP activity up to > 30% and mineralization activity up to > 100%. Thus, the EtOAc fraction shows osteogenesis activity through stimulating activities of the two markers for bone generation including ALP and mineralization in osteoblast cells. The fraction is now under extensive investigation to isolate and fully understand the modes of action of the active compounds.

Keywords: *Clinacanthus nutans*, osteoblast MC3T3-E1, osteogenesis activity.

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INTRODUCTION

Osteoporosis is a common global disease. Osteoporosis is an increase of the brittleness of the bones, leading to higher risk of fractures, thus seriously affecting the health and life of individuals and the whole society. The World Health Organization (WHO) is very concerned and wants to improve this situation to ameliorate human health and life quality. Statistics show that over 200 million people worldwide and about 2.8 million Vietnamese suffer from osteoporosis (Sözen et al., 2017).

Osteoporosis occurs due to an imbalance between bone loss and bone formation, in which bone formation occurs more slowly than bone loss. Studies focusing on the discovery of active substances that increase bone formation or reduce bone loss to treat osteoporosis are of interests (Tabatabaei-Malazy et al., 2017; An et al., 2016). In addition, the long-term use of current chemical anti-osteoporosis medications has some limitations in effectiveness as well as side effects (Tabatabaei-Malazy et al., 2017). Therefore, the discovery and use of natural substances that are able to induce new bone regeneration with less adverse effects to treat osteoporosis and maintain bone strength are a new research approach with great potential in application not only osteoporosis, but also other related diseases.

Some medicinal plants have been traditionally used for quick bone healing; shorten the treatment time for broken bones and safe. These medicinal plants will be the potential sources for screening and researching of substances that induce new bone regeneration activity. *Clinacanthus nutans* is a traditional herbal medicine to treat fractures in some Asian countries like China and Vietnam. The tree popularly grows in many provinces of Vietnam such as Thanh Hoa, Hoa Binh, Lam Dong and Tay Ninh... Studies on the chemical composition of this plant showed that it contains various groups of compounds such as flavonoids, steroids, triterpenoids, cerebrosides, glycosylglycerolipids, glycerides, glycosides containing sulfur

(Tu et al., 2014, Kamarudin et al., 2017; Mai et al., 2016). The isolated substances have different biological activities such as antioxidant, antiviral, anti-inflammatory, anti-infection and anti-angiogenesis. Although *C. nutans* has long been used as a traditional medicine to hasten fracture healing, the intensive study of bone regeneration inducers in this plant has not yet been reported. We think that *C. nutans* may contain substances capable of inducing new bone regeneration. In this paper, the osteogenesis activity of some extracts from *C. nutans* was evaluated using an osteoblast cell line. The results are promising for further studies on the isolation of desired substances and molecular mechanisms of action, as well as for prediction/confirmation of practical application for fractures.

MATERIALS AND METHODS

Plant samples were collected in Da Lat, Lam Dong Province in October 2019, stored and identified by Dr. Nguyen Thi Thanh Huong, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology.

Pre-osteoblast cell line MC3T3-E1 were purchased from Sigma (USA). Minimum Essential Medium Eagle-alpha modification (Alpha MEM) cell culture medium and fetal bovine serum (FBS) were purchased from Invitrogen (USA). Alizarin red S (anthraquinone derivative), p-NPP and MTT were purchased from Sigma (USA). The TLC 60 F₂₅₄ silica gel pre-coated plates (20 × 20 cm) were purchased from Merck (Germany). Other chemicals are all in analytical grade.

Preparation of the plant extracts

The stems and leaves of *C. nutans* (10 kg) were dried at <50°C, then grinded into a fine powder. The powder was then extracted with ethanol (EtOH) by immersion method at room temperature for two weeks. The extract was then collected by filtration through a filter paper and removal of the solvent under low pressure (<50 atm) with a rotary evaporator to obtain ethanol residue. The ethanol residue was dissolved in water and extracted successively using various organic solvents

including n-hexane, ethyl acetate (EtOAc) and n-butanol (BuOH), to obtain three fractions for further experiments.

Separation of components by thin layer chromatography (TLC)

The analysis was carried out on a silica gel prepared aluminum plate with a thickness of 1 mm (Silica gel 60, F₂₅₄, Merck). The analytical solvent systems used include: i) hexane: acetone at the ratio of 8.0 : 2.0; ii) toluene : ethyl acetate : acetone : formic acid (TEAF) at a ratio of 5 : 2 : 2 : 1; and iii) dichloromethane (DCM) : MeOH in the ratio of 8:2. The plate was stained with a solution containing cesium chloride and ammonium molybdate.

Evaluation of bone regeneration induction activity of the fractions

MC3T3-E1 cells were cultured in alpha-MEM medium supplemented with 10% FBS at 37°C in a 5% CO₂ incubator. Cell differentiation was performed in an osteogenic differentiation medium (ODM) containing alpha-MEM supplemented with 5% FBS 1% penicillin-streptomycin, 100 µg/mL ascorbic acid, 10 nM dexamethasone and 10 mM β-glycerophosphate.

Cell viability was assessed by MTT method. Cells were cultured in 96 well plates at a density of 5×10^3 cells/well under the presence of the testing substances at concentrations of 5, 10, 25 and 50 µg/mL. The negative control was DMSO solvent. After incubation for 24 hr, the cells were washed and 100 µl MTT (1 mg/mL) was added to the wells. The plate was incubated further 4 hr. Finally, DMSO (150 µl) was added and the absorbance was measured at A₅₄₀ nm. Based on the MTT results, the concentrations that did not affect the cell survival were selected for subsequent *in vitro* studies to assess the effect of the test samples on the differentiation of bone-forming cells (Nguyen et al., 2011, 2013, 2014).

Evaluation of alkaline phosphatase activity (ALP)

Alkaline phosphatase (ALP) activity was determined using previous methods of

Nguyen et al. (2011, 2014). To evaluate ALP activity, MC3T3-E1 cells were cultured in 24 well plates in alpha-MEM medium supplemented with 10% FBS for 1 day. The culture medium was then replaced with ODM medium containing test samples at concentrations of 5, 10, 25 and 50 µg/mL or DMSO (control) and the plate was incubated for further 6 days. The cells were then incubated for 1 hr at 37°C in 250 mM carbonate buffer containing 1.5 mM MgCl₂ and 15 mM p-NPP. ALP activity in samples was measured at 405 nm using a spectrophotometer. ALP activity was calculated based on the formula:

$$ALP (\%) = \frac{A - A_o}{A_o} \times 100\%$$

Where: A was the absorption of the cells supplemented with the test sample, and A_o was the absorption of the cells without test sample.

Evaluation of bone mineralization

The level of mineralization of osteoblast cells was determined by staining the cells with alizarin red-S in a 6-well plate as described previously (Nguyen et al., 2011, 2013, 2014; Park et al., 2016). The cells were cultured at a concentration of 1×10^5 cells/well in alpha-MEM under the presence of test samples at concentrations of 5, 10, 25 and 50 µg/mL or solvent (DMSO) alone. 100% ethanol was used to fix the cells and then the plate was stained with alizarin red-S 40 mM (pH 4.2) for 1 hour. The cells were washed for 15 minutes with 10% cetylpyridium chloride and dissolved in 10 mM sodium phosphate buffer (pH 7.0). The staining of the cells shows the level of mineralization and the optical density is measured at 562 nm. Bone mineralization activity was calculated based on the formula:

$$Mineralization (\%) = \frac{A - A_o}{A_o} \times 100\%$$

Where: A was the absorption of the cells supplemented with the test substance, and A_o was the absorption of the cells without the test sample.

Statistical analysis

The data were statistically analyzed using t-test or ANOVA. The difference between the samples was considered to be significant when P value is <0.05.

RESULTS AND DISCUSSION

Phytochemical screening of *C. nutans* fractions

The phytochemicals in the fractions of *C.*

nutans were analyzed qualitatively by thin layer chromatography in three different solvent systems, as a basis for the isolation of active substances. The results are shown in Figure 1. The chromatogram combined with staining using specific dyes suggested that EtOH, hexane, and EtOAc fractions mainly contained flavonoids, alkaloid, coumarin and glycoside groups, while the BuOH fraction mainly contained flavonoid group (data not shown).

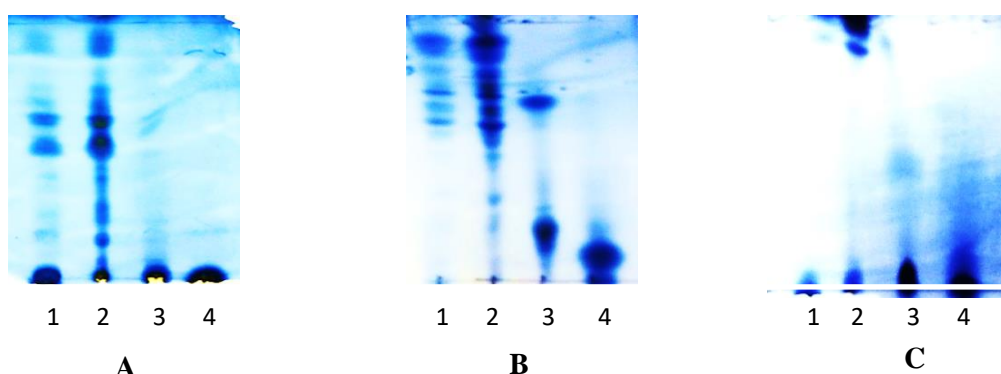


Figure 1. TLC chromatogram of *C. nutans* fractions. A. hexane : acetone (5:2); B. toluene : ethyl acetate: acetone: formic acid (5:2:2:1); C. DCM : methanol (8:2). 1. ethanol fraction; 2. hexane fraction; 3. ethyl acetate fraction; 4. n-butanol fraction

The viability of osteoblast when treated with fractions

The results in Figure 2 show that the samples at concentrations of 5, 10, 25 and 50

$\mu\text{g/mL}$ were non-toxic to MC3T3-E1 cells. Therefore, these concentrations were selected to assess the effect of the fractions of interest on the differentiation of osteoblasts.

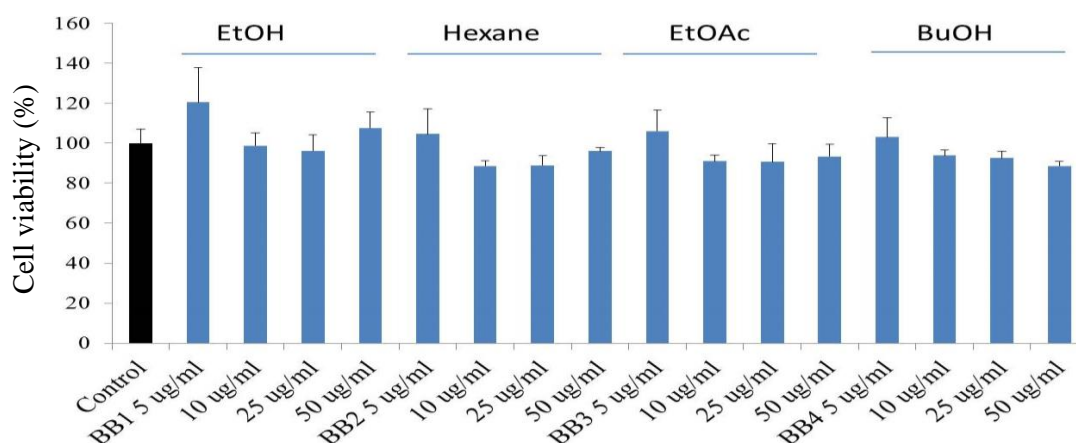


Figure 2. Cell viability of MC3T3-E1 cells under the presence of *C. nutans* fractions. DMSO (Control); Ethanol extract (EtOH-BB1); Hexane fraction (BB2); Ethyl acetate fraction (EtOAc-BB3); Butanol fraction (BuOH-BB4)

Effects of *C. nutans* fractions on ALP activity of osteoblasts

The differentiation of osteoblasts can be classified into three stages: i) cell proliferation; ii) maturation of the matrix (matrix maturation); and iii) matrix mineralization. Stage ii) is defined as the time of the maximum expression of ALP activity, which is an important indicator of bone regeneration.

The results in Figure 3 show that, among the test samples, the EtOAc and total EtOH extracts have the most pronounced effect on stimulating ALP activity, increasing from 10% (at concentrations of 5 and 10 $\mu\text{g/mL}$) to 30% (at concentrations of 25 and 50 $\mu\text{g/mL}$). These fractions were further evaluated for the effect on mineralization activity of MC3T3 cell lines.

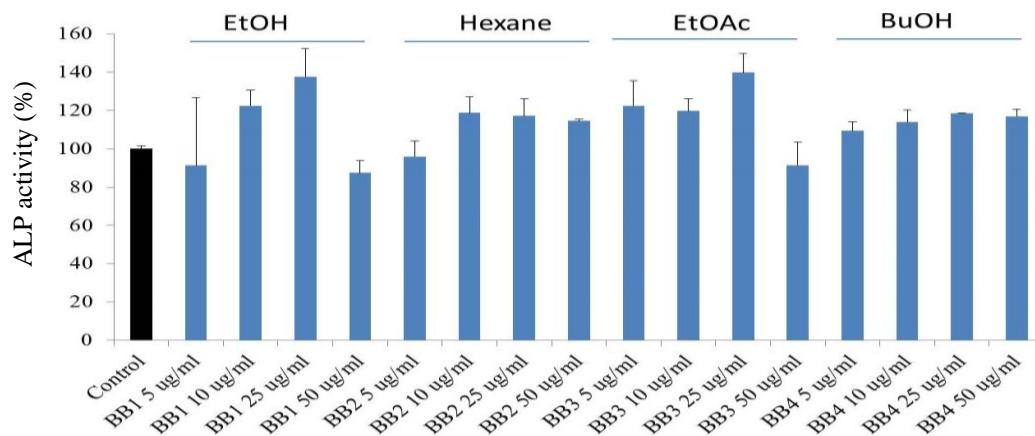


Figure 3. Effects of *C. nutans* fractions on ALP activity of MC3T3-E1 osteoblasts. DMSO (Control); Ethanol extract (EtOH-BB1); Hexane fraction (BB2); Ethyl acetate fraction (EtOAc-BB3); Butanol fraction (BuOH-BB4)

Effects of *C. nutans* EtOH and EtOAc fractions on bone mineralization activity of MC3T3-E1 osteoblast cells

The matrix mineralization stage is the third (final) stage of the osteoblast differentiation process. At the end of the mineralization, calcium deposits (bone mineralization) can be observed with appropriate staining. The results in Figure 4 show that the EtOH and the EtOAc fraction can enhance the mineralization activity significantly, up to > 100% with the EtOAc fraction. Thus, this fraction could be a potential source for the acquisition of desired substances that induce bone regeneration.

Because the currently using synthetic anti-osteoporosis drugs has some limitations and causes undesirable side effects when used over a long period. Thus, investigations on natural substances, especially phytochemicals from

traditional medicinal plants that have been historically used to treat osteoporosis and osteoarthritis diseases are urgently needed. Among the published reports, berberine has been extensively studied. This is an alkaloid compound found in the roots and stems of the Huangshi tree (*Coscinium usitatum* L.). Lee et al. (2008) showed that berberine increased the expression of a number of marker genes in bone differentiation process, including osteopontin and osteocalcin. It also enhances the activity of the main transcription factor Runx2 during bone formation. Tai et al. (2009) isolated chrysoeriol compound from leaves of Vietnamese *Eurya ciliata* Merr and found that it can stimulate proliferation of MC3T3-E1 cells at a concentration of 0.2–5.0 $\mu\text{g/mL}$. This compound also enhanced ALP activity and matrix mineralization at 5 $\mu\text{g/mL}$. However, the mechanism of action at the molecular level, as well as the full evaluation on the *in vivo* model has not been investigated yet.

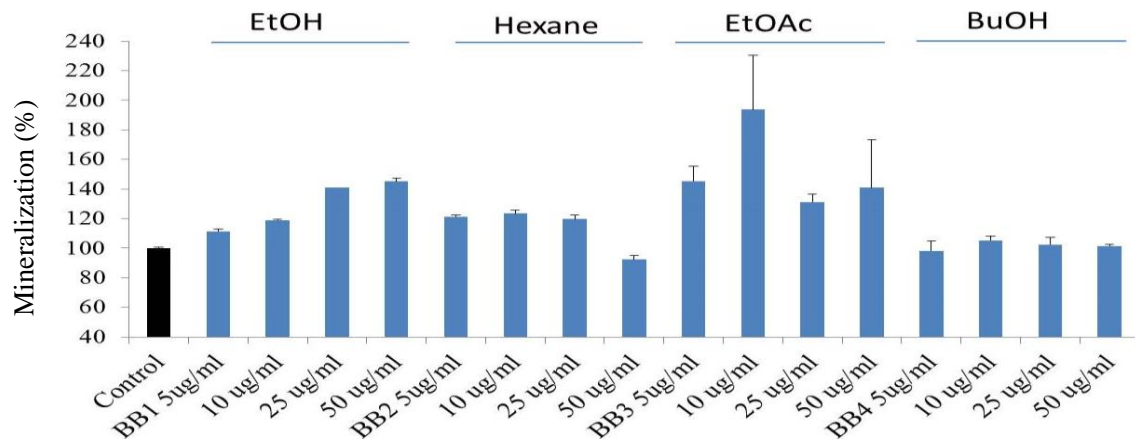


Figure 4. Effect of *C. nutans* EtOH and EtOAc fractions on bone mineralization activity of osteoblast cells MC3T3-E1. DMSO (Control); Ethanol extract (EtOH-BB1); Hexane fraction (BB2); Ethyl acetate fraction (EtOAc-BB3); Butanol fraction (BuOH-BB4)

CONCLUSION

Ethanol, hexane, ethyl acetate and butanol fractions of *C. nutans* exhibited bone regeneration activity to various extents. Among them, ethyl acetate fraction can increase ALP enzyme activity and mineralization activity of osteoblast cells up to > 30% and up to > 100%, respectively. Ethyl acetate fraction of *C. nutans* should be explored further to isolate natural osteogenic compound(s) that can induce bone regeneration for therapeutic applications.

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