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Full Proceeding Paper

ALKALINE PHOSPHATASE ACTIVITY OF *GRAPTOPHYLLUM PICTUM* AND *SPHILANTHES ACMELLA* FRACTIONS AGAINST MC3T3-E1 CELLS AS MARKER OF OSTEOBLAST DIFFERENTIATION CELLS

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ABSTRACT

The hexane, ethyl acetate, *n*-butanol and water fractions from *Graptophyllum pictum* and *Spilanthes acmella* were evaluated the stimulative activity on alkaline phosphatase (ALP) of MC3T3-E1 osteoblast cells. ALP activity is a marker of osteoblast differentiation. Among the tested, the *n*-butanol and water fractions of *Graptophyllum pictum* showed the activity to 112% and 122% respectively, otherwise the *n*-butanol and water fractions of *Spilanthes acmella* showed the activity to 126% and 127% respectively.

Keywords: *Graptophyllum pictum, Spilanthes acmella,* osteoblast cells, Alkaline phosphatase

INTRODUCTION

Osteoporosis, is a disease characterized by reduced bone mass, quality and strength, changes in skeletal micro-architecture, and increased fracture risk.[1] Osteoporosis, which literally means "porous bone," is often referred to as the silent disease because symptoms are not noticed until a fracture occurs.[2] Anabolic skeletal agents affect processes primarily associated with bone formation, and they reduce fracture incidence by improving bone qualities in addition to increasing bone mass.[3]
Osteoblast play a crucial role in bone formation through the proliferation and differentiation. Especially, osteoblast differentiation, an important process for its function, confers marked rigidity and strength to the bone while still maintaining some degree of elasticity. Thus stimulation of osteoblast differentiation has been suggested to be an important therapeutic approach for the prevention and treatment of bone disease such as osteoporosis. Osteoblastic differentiation is a complex process of sequential expression of marker proteins such as alkaline phosphatase (ALP), osteocalcin and osteopotin.[4]

In order to find the ideal anabolic agent with stimulation on alkaline phosphatase (ALP) activity as a marker of osteoblast differentiation, we carried out the screening of 32 Indonesian traditional medicinal plants (at the previous study). Based on the screening, *Barleria lupulina* aerial parts, *Graptophyllum pictum* leaf, and *Spilanthes acmella* aerial parts stimulated ALP activity to 139%, 128% and 169%, respectively.[5,6]

Graptophyllum pictum is one of popular traditional medicines extensively used in Indonesia. The leaves are used by the name of "Daun ungu" for the treatment of constipation, rheumatism, menstruations, hemorrhoid, urinary infections, scabies, swelling, maturing boil process, smooting skins, wound, dermatitis, hepatomegaly, ear disease, laxative, and chancre.[7] In addition, the leaves are popular as a folk remedy for the treatment of several conditions such as anti-fungi,[8] anti-inflammation[9] and anti-plaque.[10]

Moreover *Spilanthes acmella* had been used in swelling, arthritis, dysuria, sprain, tonsillitis aphtha, tooth-ache,[7] and as anti-infection,[11] anti-malarial,[12] anti-fungi,[13] anti-microbial,[14,15] anti-inflammation and analgesic.[16]

In this paper, we reported alkaline phosphatase stimulatory activity of the 70% ethanol extracts, hexane, ethyl acetate, *n*-butanol and water fractions of *Graptophyllum pictum* and *Spilanthes acmella* against MC3T3-E1 osteoblast cells.

MATERIALS AND METHODS

Materials

Osteoblast-like cell MC3T3-E1 was purchased from Riken Cell Bank, Tsukuba, Japan. Fetal bovine serum (FBS) was purchased from JRH Bioscience. Alpha modification of Eagles medium (α-MEM) was obtained from MP Biomedicals, Inc. (Ohio, USA). Streptomycin sulfate salt and penicillin G were purchased from Sigma Chemical Co. Trypsin (0.25%) was purchased from GIBCO. Dulbecco`s PBS (-) was purchased from Nissui Pharmaceutical Co., Ltd.. Sodium dodecyl sulfate and disodium *p*-nitrophenyl phosphate hexahydrate were purchased from Wako Pure Chemical Industries. Albumin standard was purchased from PIERCE, USA. All other reagents were from Wako Pure Chemical Industries, Ltd.

Cell culture

MC3T3-E1 cells were cultured in 50 mL tissue culture flask in α-MEM containing 10% FBS in a CO2 incubator at 37℃ and subcultured every 3 days at a dilution of Trypsin (0.25%). The 5 x 104 cells were seeded in 24-well plates and incubated in fresh α-MEM containing 10% FBS at 37℃ under 5% CO2 for 3 days. After 3 days incubation, the cells were washed with serum-free □-MEM and cultured for 3 days in α-MEM containing 1% FBS and serial dilutions of the samples which determined their effect on alkaline phosphatase (ALP) activity.

Alkaline Phosphatase Assay

ALP activity was assayed according to the method of Kumegawa[17,18] with slight modifications. At the end of incubation, the medium was transferred to the sterilized microtube, and it was centrifuged for 10 min at 3000 rpm. The supernatant fluid was used for the assay of LDH-cytotoxic test. The cells were washed with PBS (-), and lysated with 250 μL of 1 mM sodium dodecyl sulfate (SDS) in a microplate mixer for 30 min at room temperature. The cell lysate was incubated with 25 μL of 40 mM *p*-nitrophenyl phosphate at an alkaline condition (25 μL of 0.4 M sodium carbonate buffer, pH 10.0 and 25 μL of 4 mM magnesium chloride) for 1 h at 37℃, and the reaction was stopped by adding 50 μL of 0.5 N NaOH. The amount of *p*-nitrophenol liberated was measured by microplate reader at 450 nm. One unit of enzyme was defined as the activity causing release of 1 nmol of product per min under the standard assay condition used. ALP activity was expressed as unit per mg protein. The protein content in the cell lysate was estimated by the BCA method with bovine serum albumin as standard.

Plant material

The leaves of *Graptophyllum pictum* and *Spilanthes acmella* were collected from Botanical Garden, Purwodadi, East Java, Indonesia in July 2007. A voucher specimen (TMPW 25663 and 25668) was preserved in the Museum of Materia Medica, Analitycal Research Center for Ethnomedicines, Institute of Natural Medicine, University of Toyama, Japan.

Extraction and isolation procedure

The leaves of *Graptophyllum pictum* and *Spilanthes acmella* (50 g) were extracted with 70% EtOH-H2O. The EtOH extract (5 g) was

sequentially extracted with hexane, ethyl acetate, *n*-butanol and water.

Preparation of test samples

The 70% EtOH extract, hexane, ethyl acetate, *n*-butanol and water fractions (1 mg) was mixed with 10 L of DMSO and was vigorously shaken for 30 minutes at room temperature. The mixture was diluted with α-MEM containing 1% FBS to the final concentration (50 μg/mL) which used for ALP stimulatory activity. The final concentration of DMSO was 0.05%.

Statistical Analysis

The results were analyzed by the *t*-test after ANOVA. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

The 70% EtOH extract of the leaves of *Graptophyllum pictum* and *Spilanthes acmella* showed ALP stimulatory activity (128% and 169% respectively) against MC3T3-E1 osteoblast cell at 50 μg/mL (Fig. 1).

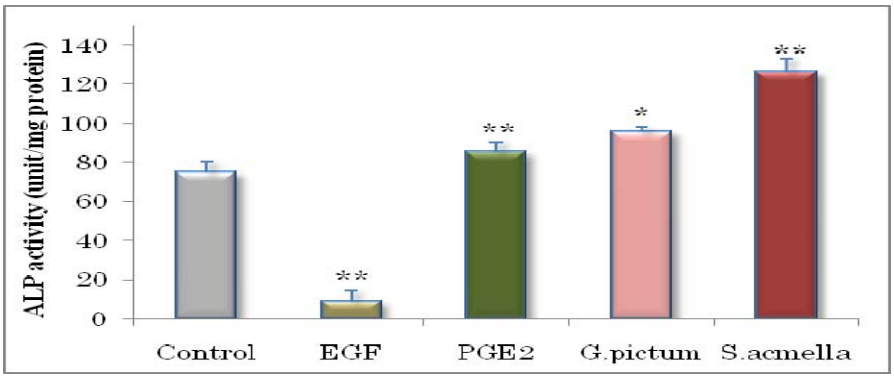
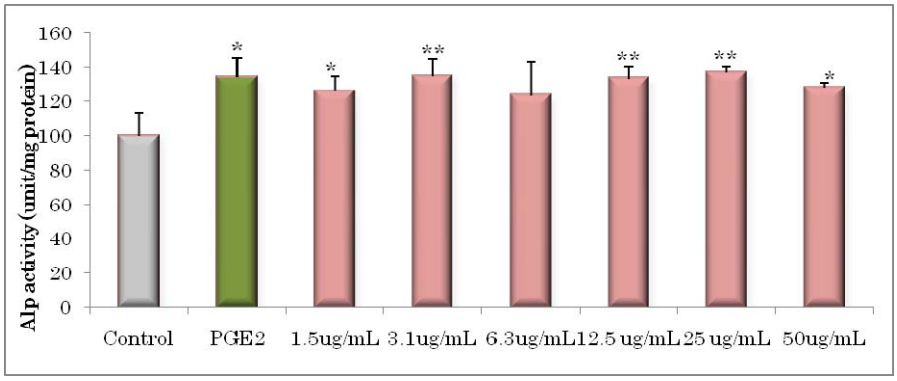


Fig. 1: Effect of 70% EtOH extract of the leaves of *Graptophyllum pictum* and *Spilanthes acmella* on alkaline phosphatase activity in MC3T3-E1 osteoblast cells at 50 μg/mL. EGF, Epidermal Growth Factor, is negative control at 10 μg/mL and PGE2, Prostaglandin E2, is positive control at 12.5 ng/mL. The value is expressed as the mean ± SD, n = 4. Significant differences in compared with control, *$p < 0.05$, **$p < 0.01$.

(a)



(b)

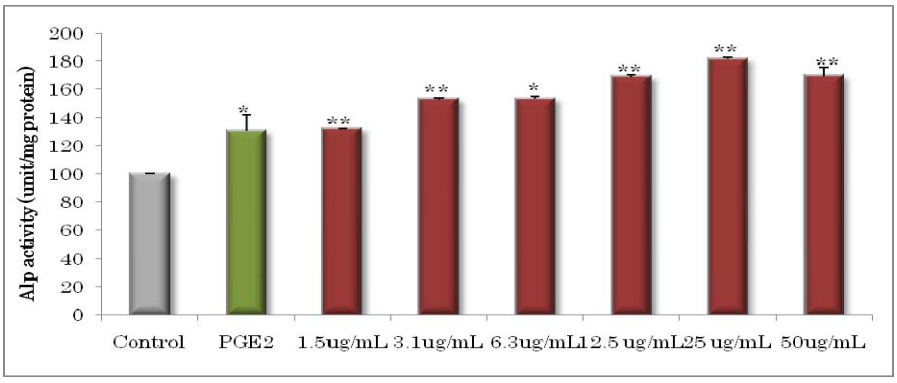


Fig. 2: Effect of 70% EtOH extract of the leaves of (a) *Graptophyllum pictum* and (b) *Spilanthes acmella* on alkaline phosphatase activity in MC3T3-E1 osteoblast cells at different concentrations (1.5 - 50 μg/mL) The value is expressed as the mean ± SD, n = 4. Significant differences in compared with control, *$p < 0.05$, **$p < 0.01$.

Figure 2 shows the effect of the 70% EtOH extract of the leaves of *Graptophyllum pictum* and *Spilanthes acmella* on ALP activity in MC3T3-E1 osteoblast cells at the different concentrations. This extracts had a dose-dependent stimulatory ALP activity to 25 g/mL.

The effect of the 70% EtOH extracts on ALP activity in MC3T3-E1 osteoblast cells suggested that the extracts have biphasic effects on osteoblast cells. At low concentrations, they stimulated ALP activity. Feng reported that enterolactone and enterodiol have biphasic effects on the viability and ALP activity of MG-63 cells and DNA synthesis in MCF-7 (breast cancer) cells. At relatively low concentrations, some phytoestrogens express an estrogenic activity and stimulate cell growth, while at higher concentrations the same phytoestrogens appear to be antiestrogenic and suppress cell growth. The biphasic mode of action may represent a viable anabolic therapy for osteoporosis .[19]

Thus, the 70% EtOH extracts were separated into hexane, ethyl acetate, *n*-butanol and water fractions. Among them, the *n*-butanol and water fraction stimulated ALP activity, but hexane and ethyl acetate did not show the activity (Fig. 3).

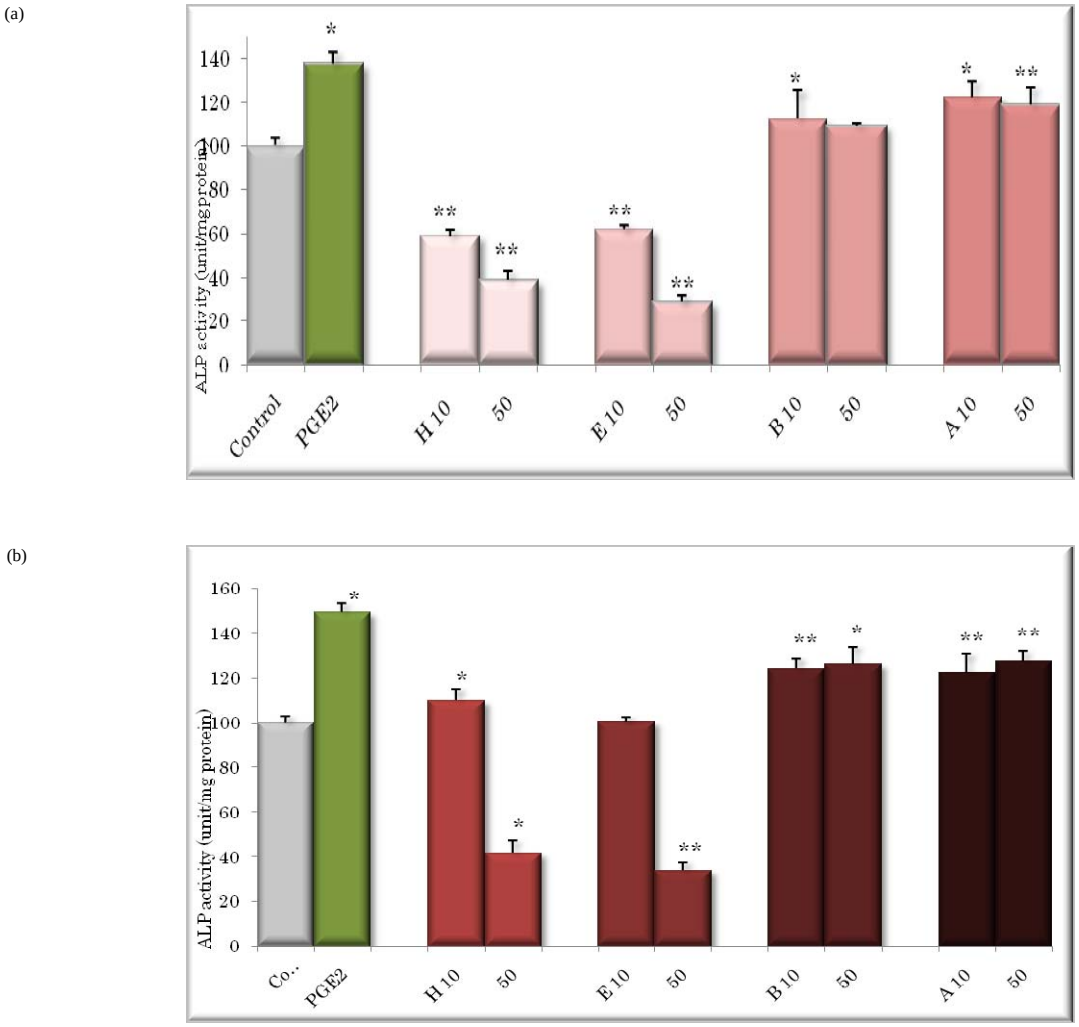


Fig. 3: Effect of hexane (H), ethyl acetate (E), *n*-butanol (B) and water (A) fraction of the leaves of (a) *Graptophyllum pictum* dan (b) *Spilanthes acmella* on alkaline phosphatase activity in MC3T3-E1 osteoblast cells. Each fraction represents at concentration between 10 and 50 µM (Co, control; and PGE2, prostaglandin E2, 12.5 ng/mL). Each value is expressed as the mean ± SD, n = 4. Significant differences in compared with control, *$p < 0.05$, **$p < 0.01$.

The *n*-butanol and water fractions of *Graptophyllum pictum* showed ALP stimulatory activity (112% and 122% respectively) against MC3T3-E1 osteoblast cell at 10 and 50 µg/mL (Fig. 3a). Otherwise the *n*-butanol and water fractions of *Spilanthes acmella* showed 126% and 127% respectively (Fig. 3b).

The *n*-butanol and water fractions of *Graptophyllum pictum* and *Spilanthes acmella* are active fraction because they stimulated ALP activity. The value of ALP activity of *n*-butanol and water fractions of *Spilanthes acmella* was higher than *Graptophyllum pictum.* So *Spilanthes acmella* had better activity than *Graptophyllum pictum* but it had a lower activity than positive control (PGE2).

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