

## RESEARCH ARTICLE

**Anti-Osteoporosis Potencies of *Zingiber officinale* Rosc. Rhizome Water Extract and DFA III Produced from *Dahlia spp. L.*: *in vivo* and *in vitro* Studies**

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**Abstract**

**BACKGROUND:** *Zingiber officinale* Rosc. is estrogenic and thus can be developed as an anti-osteoporosis. Difructose anhydride III (DFA III), possesses anti-osteoporosis potencies. This study aimed to investigate the anti-osteoporosis activity of ginger rhizome water extract (GE) and DFA III from dahlia tubers in ovariectomized (OVX) rat models and to determine their anti-osteoclastogenic effect *in vitro*.

**METHODS:** This study was conducted using 25 female rats. Blood sampling was carried out at the beginning and end of treatments. Femur bones were isolated after daily 14-day treatments, measured for density, and processed for histological staining. RAW 264.7 cells were induced by osteoclast differentiation factor. A cell viability assay was employed to determine the cytotoxicity of DFA III and GE. The inhibition of osteoclastogenesis was investigated by tartrate-resistant acid phosphatase staining.

**RESULTS:** All groups showed no difference in body weight elevation and serum lipid profiles. The GE and DFA III caused no effect on bone density. However, the GE or DFA III groups showed higher osteoblast numbers compared with the control groups. A significantly less osteoclast was found in the GE+DFA III group. The GE and DFA III showed no toxicity on RAW 264.7 cells. GE showed strong inhibitory effects on the post stimulation osteoclastogenesis model. The combination of GE and DFA III was synergistic in reducing the osteoclastogenesis confluency in RAW 264.7 cells.

**CONCLUSION:** The data support our hypothesis that GE and DFA III can decrease the risk of osteoporosis by osteoclastogenesis inhibition.

**KEYWORDS:** *Dahlia spp.*, estrogenic, ginger, osteoclast, osteoporosis, ovariectomy, RAW 264.7 cell

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**Introduction**

Estrogen is a hormone that not only plays an important role in growth, differentiation, and reproductive system but also has other physiological functions, such as bone mass defense and cardiovascular protection.(1) Estrogen deficiency increases the risk of various health problems, such as hot flushes, sleep disturbances, vaginal dryness, joint pain, mood swings, osteoporosis, and cardiovascular

diseases.(1) Therefore, estrogen deficiencies need to be managed to improve the quality of life.

Osteoporosis caused by estrogen deficiency is one of the health problems that greatly affect a person's quality of life. Osteoporosis is a common health problem faced by developing and developed countries. The hospitalization caused by osteoporotic fractures in the United States for 55 years-old women and older is greater than that of other serious illnesses such as cardiovascular diseases and breast cancer.(2) In Indonesia, the prevalence of osteopenia, early

osteoporosis, in women above 55 years old is 6 times higher than that of men and the increase of osteoporosis incidence is twice in women.(3) Given this information, an effort to prevent osteoporosis is vital, especially for women.

Osteoporosis or bone loss occurs due to the imbalance between the rate of bone formation by osteoblasts and bone resorption by osteoclasts, a process known as bone remodeling. Estrogen deficiency plays a role in the bone remodeling mechanism and pathogenesis of osteoporosis by modulating the production of cytokines and growth factors in the bone marrow and bone cells.(4) Among these cytokines, interleukin-1 (IL-1) and tumor necrosis factor are the dominant cytokines that stimulate bone resorption and inhibit bone formation.(4) The mechanisms of IL-1 and tumor necrosis factor (TNF) in these effects include stimulation of the proliferation of osteoclast precursors and inhibition of osteoclast apoptosis. A significant increase in IL-1 and TNF production has been observed in estrogen deficiencies.(4)

Hormone replacement therapy (HRT) is commonly used to treat hormone deficiency. However, estrogen HRT causes various side effects, including an increased risk of developing breast and endometrial cancer due to the nonselective action of estrogenic agents.(1) On the other hand, numerous plants are used as natural medicine. Natural compounds from plants that have estrogen-like activity (estrogenic) are an alternative in overcoming menopausal health problems. In general, natural estrogenic compounds are not as potent as synthetic estrogens but are safer because of the lower side effects.(1,5) Currently, a widespread interest focuses on the use of natural resources for the treatment of estrogen deficiencies, including the reduction of osteoporosis risk.

Ginger (*Zingiberis officinale* Rosc.) is one of the natural ingredients that shows estrogenic properties. (1,5,6) Ginger is considered a safe herbal remedy with minimal or insignificant side effects. The main pharmacological activities of ginger and its isolates include immunomodulator, antitumor, anti-inflammatory, anti-apoptotic, antihyperglycemic, antilipidemic, and anti-emetic effects.(7) Ginger is a plant that retains a significant estrogenic effect.(1,5) *In vivo*, ginger rhizome extract increases uterine weight after being given for 3 days in young mice.(6) Associated with osteoporosis, ginger rhizome extract also induces bone formation by osteoblasts.(8)

Difructose anhydride III (DFA III), a functional oligosaccharide, has beneficial effects on the prevention of osteoporosis by increasing calcium absorption and bone density, as proven by the numbers of *in vivo* and clinical

studies as previously summarized.(9) DFA III can be produced enzymatically from fresh dahlia (*Dahlia spp.* L.) tubers, which are abundantly found in Indonesia.(10) Locally produced DFA III can increase the commercial benefit of Indonesian natural resources and be developed as functional foods or food supplements (11) to reduce the osteoporosis risk.

The individual positive effect of ginger rhizome water extract (GE) (8) and DFA III (9) in preventing osteoporosis by inducing bone formation or by strengthening the bone density via increasing mineral uptake, respectively, has been recognized. Thus, a combination food supplement formula of those two has been proposed aiming to achieve better performance.(9) However, their effect, either in single or in combination, on osteoclastogenesis remains unknown. In this study, we aimed to examine the anti-osteoporosis potency of GE and DFA III prepared from dahlia tubers (referred to as DFA III from hereafter), single or in combination, by using *in vivo* and *in vitro* approaches. Ovariectomized (OVX) rats were used as a model for estrogen deficiency/high-osteoporosis risk condition, whereas RAW 264.7 cells were used as an osteoclastogenesis model. We verified that although GE and DFA III caused no effect on the bone density, histological and cellular analyses revealed that GE, DFA III, or their combination can modulate the osteoblast/osteoclast number and inhibit osteoclastogenesis.

## Methods

### Material Preparation

All the materials were prepared in the Research Unit for Clean Technology, Indonesian Institute of Sciences. The “Emprit” variant of ginger (*Zingiber officinale* Rosc. var. amarum) was used in this study. Ginger rhizome slices were dried under high temperature and relatively short time and further processed into a dried form and standardized in accordance with the Indonesian Herbal Pharmacopoeia standards. The GE was prepared from the dried form following an optimized method as previously reported.(12) Based on that study, we extracted at 90°C for 30 minutes, as it is the optimum extraction condition to get the highest total flavonoid and phenolic content.

DFA III was prepared from dahlia tubers using a previously described enzymatic method.(10) The DFA III forming enzyme inulin fructo transferase (IFTase) was obtained as in previous studies.(13-15) Briefly, dahlia tubers were processed into chips and extracted at high temperatures using water as a solvent, followed

by the reaction between the aqueous extract with IFTase, purification, and crystallization to obtain pure crystalline DFA III.(10,13,14)

### **Animal Procedure and Treatment**

Before the study, young adult (6–8 weeks old) female rats (Sprague Dawley strain) were acclimatized and maintained in the Animal Facility, Integrated Research and Testing Laboratory Universitas Gadjah Mada in accordance with a standard procedure.(16) This study was approved by the animal welfare committee of Universitas Gadjah Mada (No. 00025/04/LPPT/V/2019).

A total of 25 rats were divided into five groups as follows: sham-ovariectomy control (Sham); OVX control; OVX with 750 mg/kg body weight (BW) GE treatment; OVX with 2,500 mg/kg BW DFA III treatment (17); and finally OVX with the combination of GE and DFA III treatment. The ovariectomy procedure was carried out using the established method (16,18-20) under a veterinary supervision.

GE and DFA III were freshly prepared before being given to the tested animals by diluting it in 0.5% carboxymethylcellulose sodium (CMC-Na, Sigma). The tested solution was given orally for 14 days starting 7 days after the ovariectomy procedures. The control groups also received 0.5% CMC-Na at the same time.

### **Measurement of Serum Lipids**

The animals were weighed every other day. The blood was sampled at the end of recovery phase after ovariectomy (before treatment) and at 7<sup>th</sup> and 14<sup>th</sup> day of the treatment. The sampling was carried out, and the blood was processed into serum as previously described.(16) The sera were sent to the Department of Clinical Pathology, Faculty of Medicine, Nursing, and Public Health, Universitas Gadjah Mada to measure the total cholesterol, triglyceride, and high-density lipid (HDL) by using commercial enzymatic colorimetric methods.

### **Measurement of Bone Density**

At the end of the treatment (day 22), the animals were sacrificed (20), and the femoral bones were isolated.(16) The left femur was kept at –20°C in a 2 mL microtube and protected from light until the measurement. The bone density was analyzed by using X-ray (16) at the Image Physics Laboratory, Department of Physics, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada. The X-ray images were analyzed by a densitometry analysis using ImageJ (National Institute of Health, Bethesda, MD, USA).

### **Histological Analysis**

The right femur was cleaned in a saline solution (Otsuka, Tokyo, Japan), fixed in paraformaldehyde, and then sent to the Department of Pathological Anatomy, Faculty of Medicine, Nursing, and Public Health, Universitas Gadjah Mada to be processed for histological analysis. Sections were made from the bone and stained with hematoxylin and eosin (HE). The slides were observed under a microscope using a 100× magnification objective lenses in a blind manner, followed by osteoblast and osteoclast counting. Ten visual fields were observed for each section, and the cell numbers were presented as the sum of total 10 fields. Three femur samples from each group were employed in the histological analysis.

### **Statistical Analysis**

All the data were presented as means±standard deviation (SD) in the *in vivo* study. Student's t-test (2 tail, type 3) (Excel version 16.45, 2019) was performed to determine the significance with a 95% confidence level.

### **Cell Culture**

The RAW 264.7 cell line was obtained from Dr. Norihiro Ishida-Kitagawa, Nara Institute of Science and Technology (NAIST), Japan. The cells were grown in the complete medium consisting of Eagle's Minimum Essential Medium (EMEM, Gibco, NY, USA), 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 1% non-essential amino acid (Nacalai Tesque, Kyoto, Japan), 100 U/mL penicillin, and 100 µg/mL streptomycin (Nacalai Tesque).(21,22)

To induce osteoclast differentiation, we suspended cells in the complete medium added with 500 ng/mL osteoclast differentiation factor (ODF).(23) RAW 264.7 cell differentiation toward multinucleated osteoclasts was followed-up *in vitro* over 4 days. The day when ODF was given was considered day 0 (D0). The ODF was replaced every 2 days to maintain the osteoclastogenesis stimulation signal.

### **Cytotoxicity Assay**

RAW 264.7 cells ( $5 \times 10^3$  cells/well) were grown in a 96-well plate and then incubated at 5% CO<sub>2</sub> content and 37°C for 24 h. EMEM was discarded, and the cells were treated with GE or DFA III samples dissolved in culture media with a concentration range of 2.5–2,500 µM for GE or of 25–2,500 µM DFA III for 96 h, and the medium was changed once at 48 h. After 96 h of treatment, the test samples were removed and washed with phosphate-buffered saline

once and given 10% WST-8 EMEM media and incubated for 2–4 h.(21) The metabolic reaction of WST-8 by the succinate dehydrogenase from living cells formed a soluble WST-8 formazan crystal with orange color, and it was read at a wavelength of 450 nm. The decrease in optical density resulting from the treatment with the test sample represented the decrease in cell viability. Cells incubated with the medium (untreated) were considered to have 100% cell viability. The percentage of cell viability was calculated based on the absorbance of sample-treated cells divided by the absorbance of untreated cells.

### Tartrate-resistant Acid Phosphatase (TRAP) Staining (Initiation Phase and Post-initiation Phase)

RAW 264.7 cells of ( $2.5 \times 10^4$ ) were grown in a 24-well plate and then incubated at 5% CO<sub>2</sub> content and 37°C for 24 h. EMEM was discarded, and for the initiation phase model, the cells were treated with samples (estradiol, GE, or DFA III) at the same time (D0) with osteoclastogenesis stimulation (500 ng/mL ODF). For the post-initiation phase model, the cells were treated with samples 24 h after the osteoclastogenesis stimulation (D +1). The untreated cells were cells treated with ODF only, whereas estradiol was given at 10 and 100 nM as the positive control. The concentration of DFA III

was 200 μM, and those of GE were 5 and 10 μg/mL. The cell cultures were then incubated again, and the medium was changed at day 4 (D +4). The cells were fixed and stained with a TRAP staining kit in accordance with the protocol. The cells were observed under a light microscope. The TRAP-positive cells were in red color with morphology as large multinucleated cells.(21,24)

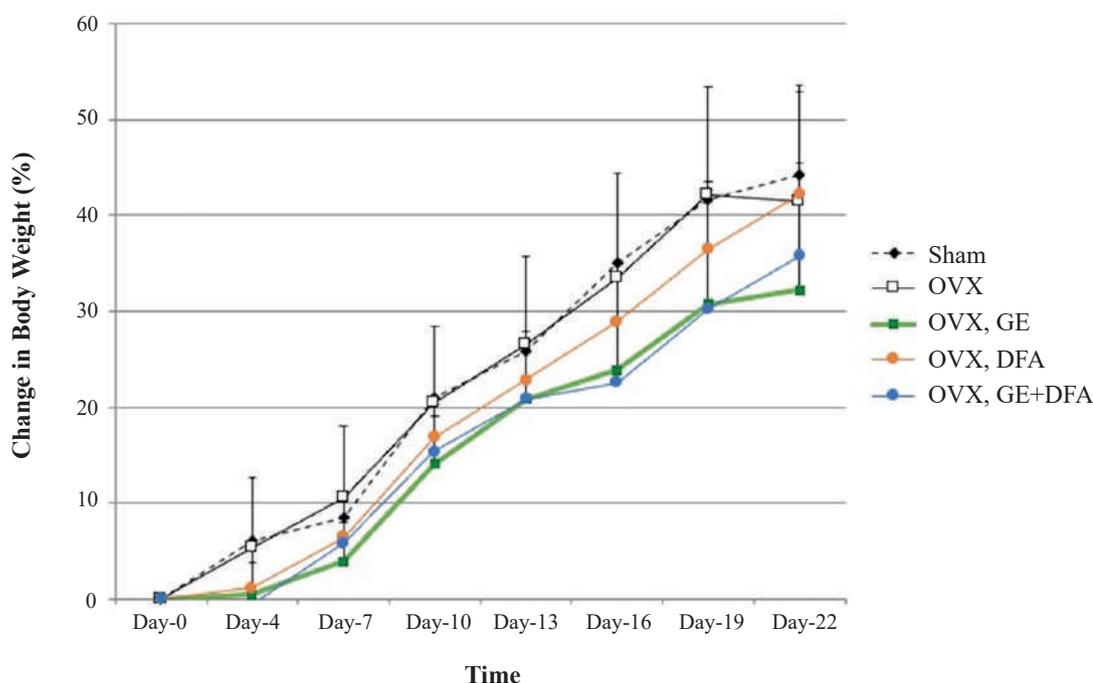
## Results

### Extraction of Ginger

GE was prepared from dried ginger following an optimized method as previously reported.(12) We extracted 10 g of dried ginger with water at 90°C for 30 minutes. The extract was then filtrated, evaporated, and dried as described in the method section until it formed a dry extract. We obtained 1.2 g (dry weight) of GE from 10 g of dried ginger.

### BW and Serum Lipid Profiles

We performed an ovariectomy procedure to mimic the estrogen-deficiency condition. Sham operating procedures were conducted to ensure that the ovariectomy itself does not cause any conditions rather than stated. The BW of the



**Figure 1. Effects of ovariectomy and sample treatments on rat BW.** Time course of BW changes in sham-operated rats (Sham; n=4), OVX rats (OVX; n=4750 mg/kg BW of GE (GE; n=5), 2500 mg/kg BW of DFA III (DFA, n=5), or combination of GE and DFA III (GE+DFA; n=5). Rats were sham-operated or OVX at day 0 and were recovered for one week. On day 7, rats were given 0.5% Na-CMC as solvent (Sham and OVX groups), or treated with GE, DFA III, or the combination of GE and DFA III diluted in solvent for each group as indicated, daily, and for 14 days. Data are expressed as means±SD. Statistical significance was determined by Student's t-test. No statistical differences were observed among groups.

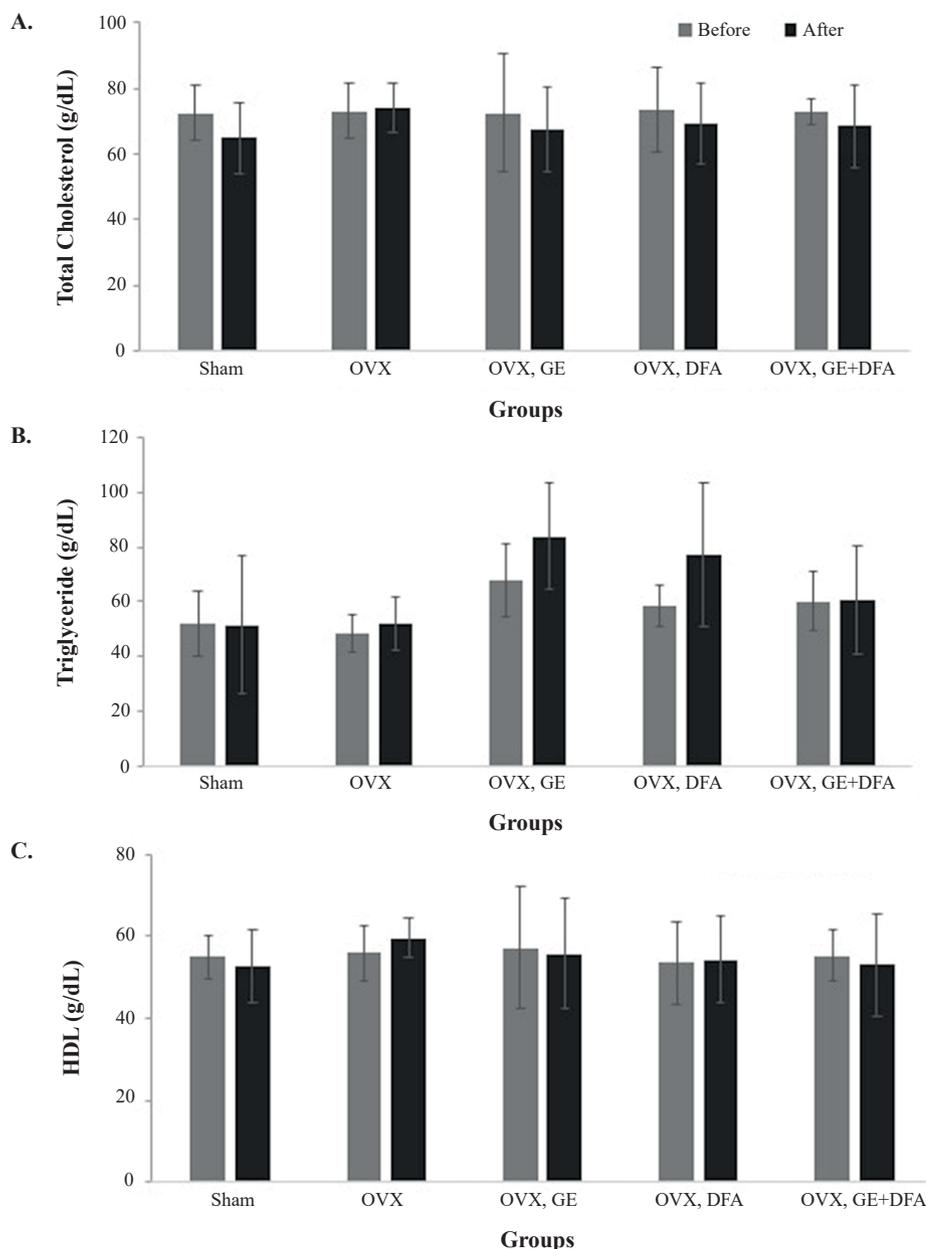
animals increased normally from day 0 (before ovariectomy) until day 22 (after a 14-day-treatment), and no significant differences were among groups (Figure 1).

Given that estrogen deficiency may affect lipid metabolism, and ginger has been reported to exhibit anti-dyslipidemic activities in rats (25,26), we also measured the serum lipid profiles. However, all groups, including GE treatment, showed no differences of the total cholesterol,

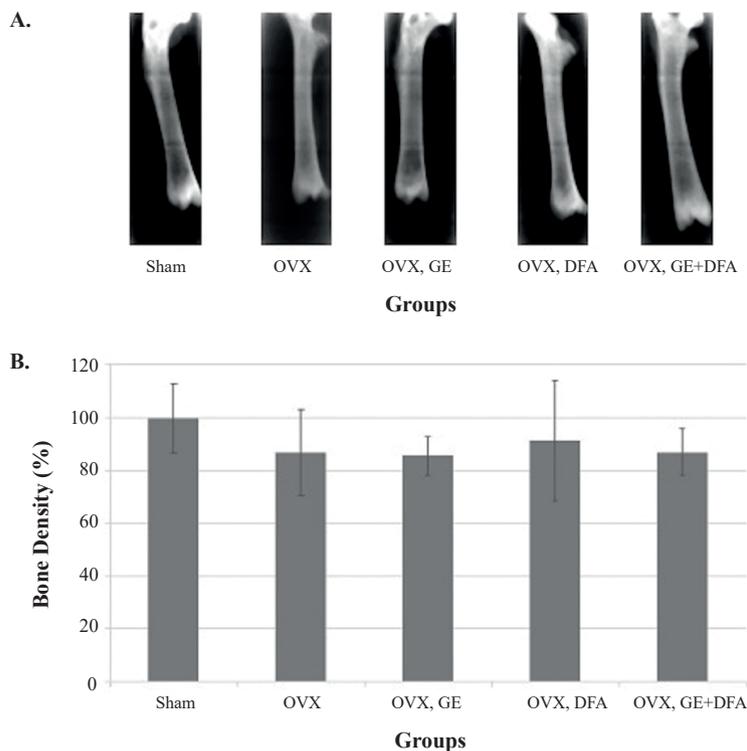
triglyceride, and HDL levels between pre/post-treatment and among groups (Figure 2). Thus, all treatments did not affect the rat BW elevation nor the serum lipid profile.

### Bone Density and Number of Osteoblasts/Osteoclasts

The OVX rat is a good model for osteoporosis conditions. (18) Therefore, we investigated the rat femoral bone in a close manner. Based on an imaging approach (Figure 3A),



**Figure 2. Effects of GE and DFA III on serum lipid profiles of OVX rats.** Rats were sham-operated (Sham) or OVX at day 0 and were recovered for one week. On day 7, rats were given 0.5% Na-CMC as solvent (Sham and OVX groups), or treated with 750 mg/kg BW of GE, 2500 mg/kg BW of DFA III (DFA), or the combination of GE and DFA III (GE+DFA) diluted in solvent for each group, as indicated, and daily for 14 days. Before and at the end of treatments, blood was sampled, and serum lipid parameters were measured. Data are expressed as means±SD (n=5). Student's t-test was carried out to compare the changes in each group and among them, showing no statistical differences.



**Figure 3. Effects of GE and DFA III on rat bone density.** Rats were sham-operated (Sham) or OVX on day 0 and were recovered for one week. On day 7, the rats were given 0.5% Na-CMC as a solvent (Sham and OVX groups) or treated with 750 mg/kg BW of GE, 2500 mg/kg BW of DFA III (DFA), or the combination of GE and DFA III (GE+DFA) diluted in solvent for each group as indicated and daily for 14 days. At the end of treatments, mice were sacrificed, and the left femur was isolated. The bone density was measured using X-ray (representative figures from each group are depicted in A), and the density value was measured by densitometry (ImageJ). The density of the Sham group was set as 100%, and other groups were compared relatively to it as shown in B. Data are expressed as means $\pm$ SD (n=4 or 5). Statistical significance was determined by Student's t-test. No statistical differences were observed among groups.

the semi-quantitative analysis revealed that bone density did not change significantly among all groups (Figure 3B). Based on histological analysis using simple HE staining (Figure 4A), we observed that the GE and DFA III groups had more osteoblasts compared with both control groups (Sham and OVX) (Figure 4B). Significantly fewer osteoclasts were found in the GE and DFA III combination group than the Sham and OVX control groups (Figure 4B). These findings support our hypothesis that GE and DFA III have anti-osteoporosis potencies by modulating bone remodeling via osteoblasts and osteoclasts. Hence, we performed further experiments by using an *in vitro* system, namely, RAW 264.7 cells, a precursor of osteoclast.

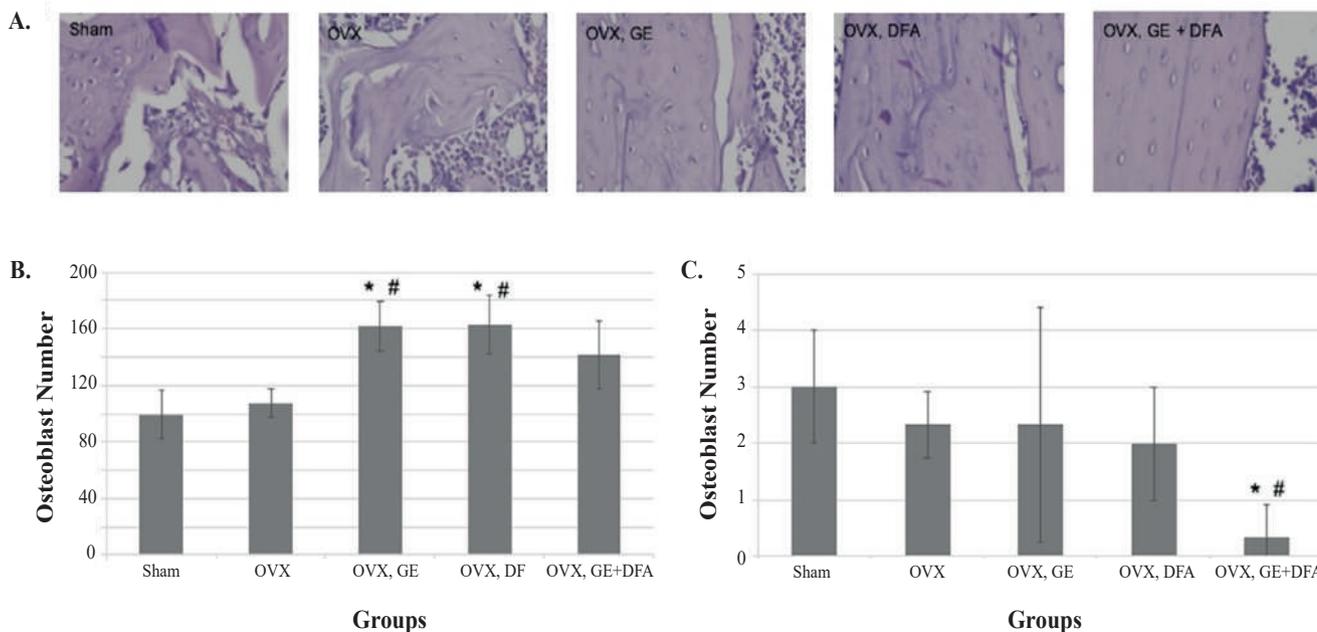
#### Cytotoxicity Assay

We evaluated the cytotoxicity of GE and DFA III to assure their safety toward cells. The RAW 264.7 mouse macrophage cell line was grown in the presence of GE (2.5–2,500  $\mu$ M) or DFA III (25–2,500  $\mu$ M) for 4 days (96 h). WST-8 assay was carried out to determine the viability of RAW 264.7 cells after exposure to GE or DFA III. The

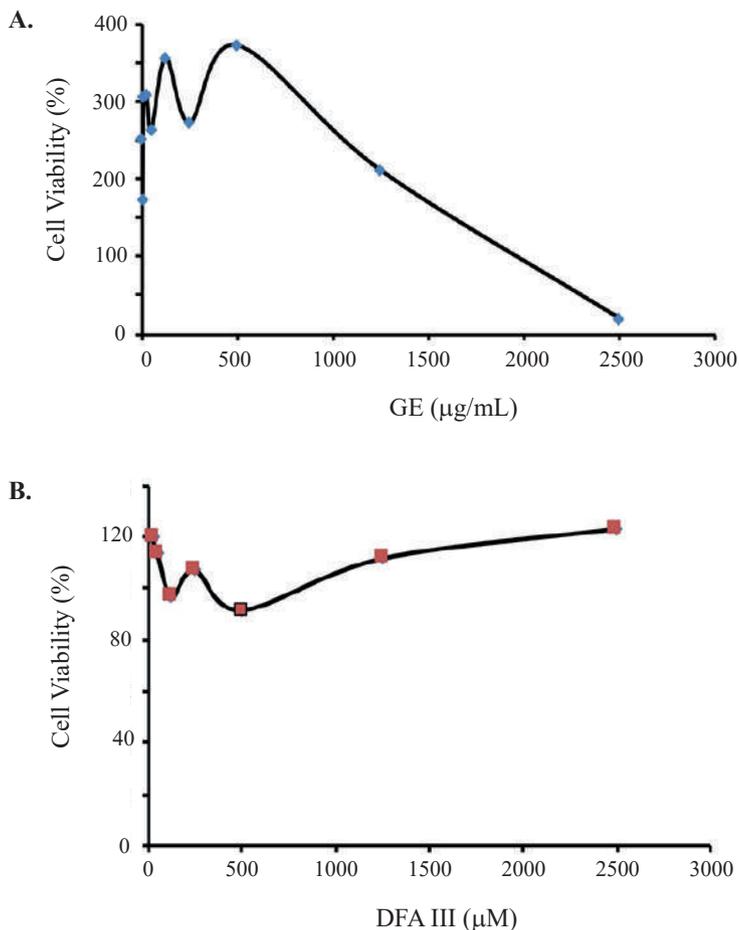
cells were viable (90%) at 2.5–2,500  $\mu$ M GE and 25–2,500  $\mu$ M DFA III (Figure 5). Moreover, the GE treatment of up to 250  $\mu$ g/mL induced RAW cell proliferation, whereas at high doses, it induced cell death. The results indicated that the extracts were nontoxic to RAW cells, with the IC<sub>50</sub> of both samples measuring more than 100  $\mu$ g/mL.

#### Osteoclast Differentiation Inhibition Test (TRAP Staining Assay)

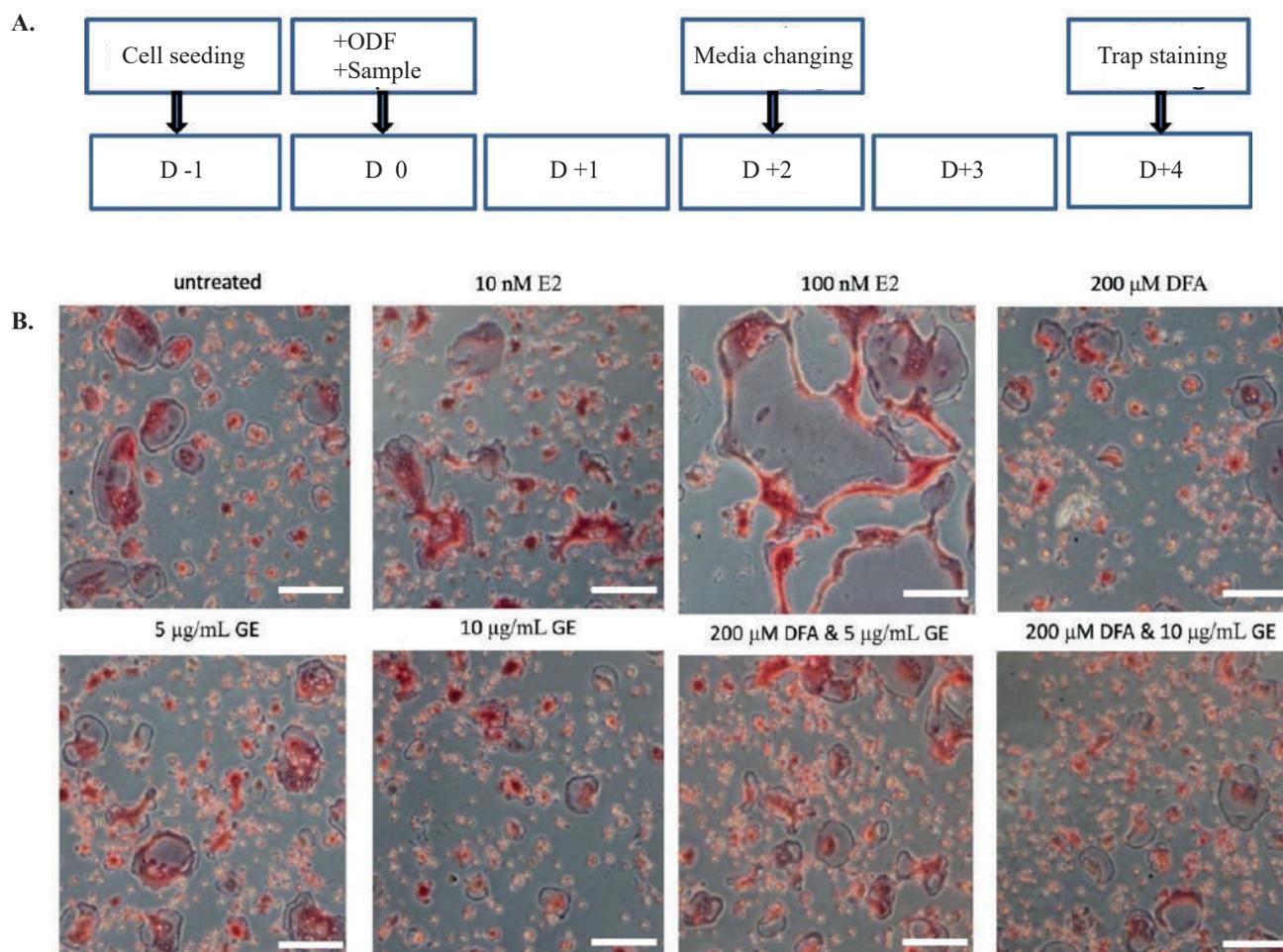
Different profiles of the inhibition of osteoclast differentiation were observed at the initiation and post-initiation stages. TRAP was expressed in high amounts by osteoclasts. Thus, we performed TRAP staining to test the inhibition of osteoclast differentiation in this study. Positive TRAP cells absorbed red color and contained numerous nuclei (equal or more than 3). Figure 6 (initiation phase) and 7 (post-initiation phase) present the results of TRAP staining. We used estradiol (E2) as the positive control (10 and 100 nM) and observed that E2 treatment positively induced osteoclastogenesis. On day 4 of incubation with the sample, the GE-treated cells showed no drastic decrease in



**Figure 4. Effects of GE and DFA III on osteoblast and osteoclast number.** Rats were sham-operated (Sham) or OVX on day 0 and recovered for one week. On day 7, rats were given 0.5% Na-CMC as solvent (Sham and OVX groups) or treated with 750 mg/kg BW of GE (GE), 2500 mg/kg BW of DFA III (DFA), or the combination of ginger extract and DFA III (GE+DFA) diluted in solvent for each group as indicated and daily for 14 days. At the end of treatment, the mice were sacrificed, and the left femur was isolated and processed for histological staining (representative figures from each group are shown in A). The numbers of osteoblast and osteoclast were counted manually in a blind manner from 10 fields of view for each section as presented in B and C, respectively. Data are expressed as means±SD (n=3). Statistical significance was determined by Student’s t-test. Asterisks and hashtags indicated a significant difference compared with sham-operated group (\**p*<0.05) and OVX group (#*p*<0.05).



**Figure 5. Cytotoxic effect of treatment of GE and DFA III on RAW 264.7 cells.** Cells ( $5 \times 10^3$ ) were treated with various concentration of samples as indicated in the figure for 96 h and subjected to WST-8 kit assay for 2–4 h. Then, the reaction was stopped, and the absorbance was measured and calculated as viable cells. A: GE (2.5–2,500 µg/mL); B: DFA (25–2,500 µM).



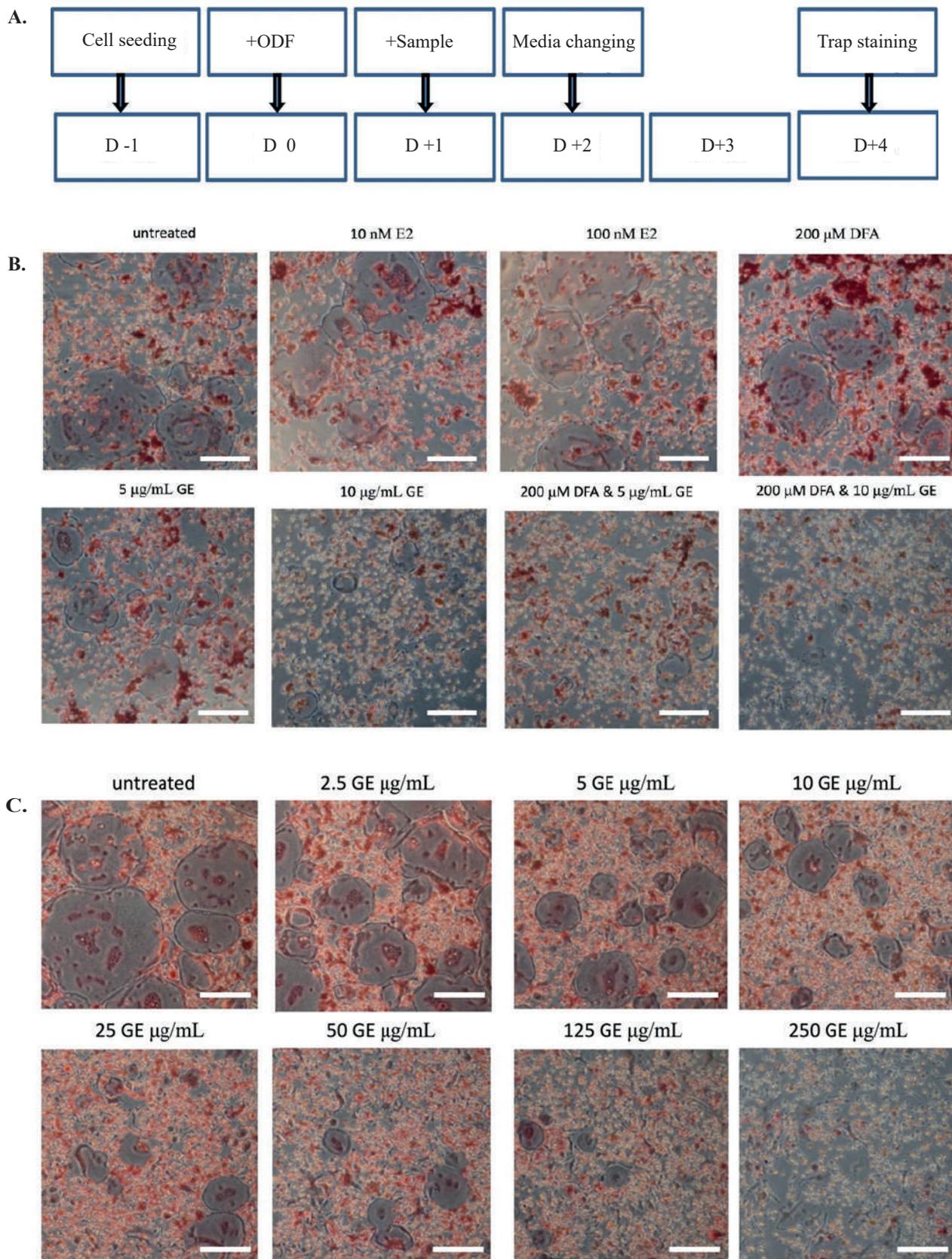
**Figure 6. Effects of DFA III (DFA) and ginger extract (GE) at the initiation phase of osteoclastogenesis.** RAW 264.7 cells ( $2.5 \times 10^4$ ) were grown in a 24-well plate for 24 h. A: Inhibition of osteoclastogenesis at the initiation phase was performed by treating the cells with samples (as indicated in the graph) and at the same time with the osteoclastogenesis stimulator (500 ng/mL ODF); B: Cells were incubated with samples for 4 days. Then, on D +4, cells were stained with TRAP staining to detect the osteoclast. TRAP-positive (osteoclast) cells were stained in red color with morphology as large and multinucleated cells. White bar: 50  $\mu$ m.

the adult osteoclast confluency compared with the untreated cells. This finding indicated that the GE did not affect osteoclastogenesis. However, the GE treatment at a dose of 250 g/mL did not show mature osteoclasts, indicating its capability to inhibit the formation of young and adult osteoclasts (Figure 7C). Furthermore, the results of the post-initiation treatment showed that after sample treatments, the cells still proliferated but did not differentiate into osteoclasts. More than 90% of the GE-treated cells (either treated with GE, DFA, or the combination) were TRAP staining negative. On the other hand, DFA III showed weak inhibition of osteoclastogenesis compared with GE. DFA-treated cells still differentiated into osteoclast. Moreover, the combination of DFA III and GE showed the dominant effect of GE because the cells failed to differentiate into osteoclast upon ODF stimulation.

## Discussion

In spite of the relative lack of effects on bone density (Figure 3), histologically, the single GE and DFA III and the combination modulated the osteoblast and osteoclast number, respectively, in OVX rats (Figure 4). To our knowledge, commonly the biological function of DFA III is reported as for modulating mineral metabolism. (17, 27-33) It has been previously confirmed that DFA III supplementation into cows' dietary does not affect osteoclast number nor osteoclastogenesis.(33) Here we show that the combination of GE and DFA III is able to reduce osteoclast number *in vivo*.

The *in vitro* model was used to confirm our observation in the *in vivo* model. Macrophage mouse cells



**Figure 7. Effects of DFA III (DFA) and ginger extract (GE) at the post-initiation phase of osteoclastogenesis.** RAW 264.7 cells ( $2.5 \times 10^4$ ) were grown in a 24-well plate for 24 h. A: Inhibition of osteoclastogenesis at the post-initiation phase was performed by treating the cells with samples (as indicated in the graph) 24 h after the cells were induced with the osteoclastogenesis stimulator (500 ng/mL ODF); B and C: Cells were incubated with samples (as indicated in figure) for 4 days. Then, on day 4, cells were stained with TRAP staining to detect the osteoclast. TRAP-positive (osteoclast) cells were stained in red color, with morphology as large and multinucleated cells. White bar: 50 μm.

(RAW 264.7) cell line served as *in vitro* model to observe osteoclastogenesis due to its ability to differentiate into osteoclast when stimulated with ODF.(21,23,24,34) The potencies of GE and DFA III for anti-osteoporosis were observed *in vivo* along with *in vitro* showed that GE and DFA III caused no effect on rat BW (Figure 1), and they were not toxic against RAW264.7 cells (Figure 5).

We designed the stimulation into the initiation and post-initiation phases to determine the mechanism of action of the samples in osteoclastogenesis. In the initiation phase, all the compounds tested (GE or DFA III) in single and combination treatments did not show the inhibition of osteoclast differentiation (Figure 6). In the post-initiation model, GE inhibited osteoclast differentiation but not proliferation (Figure 7). This phenomenon can be observed from the results of TRAP staining: GE-treated cells still proliferated but did not differentiate into osteoclasts, and the results of TRAP-negative cell staining >90%. The main compound of ginger, 6-gingerol, has been reported to inhibit osteoclast differentiation in the co-cultures of osteoblast and osteoclast cells induced by IL-1. Moreover, the osteoclastogenesis inhibitory effect of 6-gingerol was mediated by its suppression of RANKL expression in osteoblast precursor cells and not by its cytotoxicity of the compound against the co-cultures.(35) Another bioactive compound in ginger, 10-gingerol, was also reported to inhibit osteoclastogenesis both *in vitro* using RAW264.7 cells and *in vivo* using the zebrafish model. Based on gene analysis, 10-gingerol suppressed several osteoclast markers in both study models.(36) These studies are in line with our current findings that ginger contains bioactive compounds that inhibit osteoclastogenesis.

On the other hand, the weak osteoclastogenesis inhibition of DFA III indicated a different role of DFA III when combined with GE and decreased osteoclasts. DFA III may increase the absorption of GE, thus increasing the effect of GE on the inhibition of osteoclast formation in an *in vivo* model. DFA III is beneficial in preventing osteoporosis by increasing calcium absorption in humans. (27,28) A number of reports also proved that DFA III can increase rat bone density and strength (29), possibly via the increase in calcium absorption in the OVX and estrogen-deficiency models.(17,30,31) To our knowledge, our current study is the first to report the potency of locally produced DFA III.

DFA III produced enzymatically from fresh dahlia tubers can be found abundantly in Indonesia and it can increase the commercial benefit of Indonesian natural resources. Given the current regulation in Indonesia,

functional foods, such as beverages and food supplements for commercial products, are easy to develop. Instant ginger drinks fortified with DFA III have been formulated and optimized.(9) Thus, a combination of GE and DFA III has a high potency to be developed as functional foods or food supplements (11) to reduce the osteoporosis risk or prevent osteoporosis.

Nevertheless, our study had at least two shortcomings. First, ovariectomy should show a significant change in certain parameters regarding the study compared with the Sham operation. For example, the level of estrogen can be measured to ensure the OVX rat as an estrogenic-deficiency model. In our study, the bone density remained unchanged. Possibly, the duration of the experiment can be extended to allow a more thorough observation. Additionally, a positive group control can be included by giving estradiol to OVX rats. The OVX models can be extended to portray other tissues, such as uterine or mammae, that are affected by estrogen deficiency.(19,20,37) Second, we focused on observing osteoclasts. *In vivo*, osteoblasts were affected positively by GE and DFA III. Therefore, further investigation in bone formation by osteoblasts and bone resorption by osteoclasts and the balance between them may be promising.

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## Conclusion

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The GE and DFA III were not cytotoxic against RAW264.7 cells. DFA III had a weak osteoclastogenesis inhibitory activity in RAW264.7 cells compared with GE, which showed a strong inhibitory effect on post-stimulation model osteoclastogenesis. The combination of GE and DFA III was synergistic in reducing the osteoclastogenesis confluency in RAW 264.7 cells and osteoclast number in *in vivo* model. Altogether, the data support our hypothesis that GE and DFA III can decrease the risk of osteoporosis by osteoclastogenesis inhibition. Based on these results, the combination of GE and DFA III has the potential to be developed as a prevention of osteoporosis.

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## Authors Contribution

MI was involved in the conceptualization, planning *in vivo* experimental design, supervising, performing experiment and the analysis for *in vivo* works, *in vivo* data curation, drafting of the manuscript, and designing of the figures. YE was involved in the experiment and the analysis of *in vitro* works, manuscript drafting. ESE was involved in the material preparation. SP was involved in the conceptualization, material preparation, and supervising. EM was involved in the conceptualization, planning of experimental designs, supervising, and giving the final approval for submission. RIJ was involved in the conceptualization, planning experimental designs, supervising, performing the analysis for *in vitro* works, *in vitro* data curation, and drafting of the manuscript. All authors discussed the results and commented on the manuscript.

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