Exploration of several plants from Baung Forest on bone formation cell models

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Submission date: 01-Nov-2021 11:44AM (UTC+0800)
Submission ID: 1689593749
File name: C-31 - J Basic Clin Physiol Pharmacol_naskah.pdf (1,007.19K)
Word count: 5007
Character count: 27978

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https://doi.org/10.1515/jbcpp-2020-0489
Received November 29, 2020; accepted March 8, 2021

Abstract

Objectives: Osteoporosis is an ailment described by a skeletal degradation of bone skeletal dominating to increases the chance of fracture. In order to find out the bone formation agents from Baung Forest plants, this research analyzed the effects of 96% ethanol extract of several plants from Baung Forest on antioxidant activity and the effect of osteoblast differentiation-related to the bone formation on the most potent extract.

Methods: The antioxidant effect and osteoblast differentiation of 96% ethanol extracts were evaluated by measuring DPPH scavenging and alkaline phosphatase in *p*-nitrophenyl phosphate effects by the enzyme-linked immunosorbent assay (ELISA) reader method, respectively.

Results: The 96% ethanol extract of *Elaeocarpus serratus* L. from Baung Forest had the strongest DPPH radical scavenging as anti oxidant (82.17%) and stimulated osteoblast differentiation (116%). Then, this extract had been fractionated based on polarity to become hexane, ethyl acetate, butanol, and aqueous fractions. All the fractions stimulated their alkaline phosphatase (ALP) activity to 138.11 ± 9.72%, 108 ± 5.05%, 148.56 ± 8.47, and 144.58 ± 1.04, respectively.

Conclusions: The extract and fractions of *E. serratus* L. can successfully inhibit DPPH radical scavenging value and increase ALP activities as markers of osteoblast functions.

Keywords: 96% ethanol extract; alkaline phosphatase; bone formation; DPPH scavenging; *Elaeocarpus serratus*.

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Introduction

Osteoporosis is an ailment described by a skeletal degradation of bone skeletal dominating to increases the chance of fracture and being a quiet ailment in many complex situations [1]. This ailment can occur because a disproportion of bone resorption relative to bone formation products in effectiveness bone equilibrium at the tissue. During growth, bone formation surpasses bone resorption, resulting in bone elaboration [2]. It is a prominent matter of elderly and estimated to increase with rising age and life span. At 1992, the 200 million global population were expected to endure from osteoporosis [3]. Then in 2000, statistical data from the International Osteoporosis Foundation represented that 1 out of 3 women over 50 years old and 1 out of 5 men will endure osteoporosis fractures for the spend of their lives [4]. This problem too occurs in Indonesia, which has reached a level of caution because the amount of osteoporosis sufferer has increased from the latest data (>19.7%). The amount of elderly in Indonesia is estimated to increase by 14% during of 1990–2025, while in the 2000–2015 period, menopausal women donated to an intensify of osteoporosis sufferers by 8.5 million [5]. WHO estimates that in 2050 the number of fracture sufferers will increase by 2 times in women and 3 times in men [6, 7].

In this study, we have found out bone formation agents from Baung Forest plants. Baung Forest is a nature tourism park with an area of 195.5 ha [8]. This forest has its natural biodiversity, beauty, and geology. In the forest, there are various types of plants that are commonly used by local residents for health therapy. The 36 plant extracts from this forest were screened for antioxidant activity (DPPH inhibition values) and the most potent extract was analyzed for its effect on osteoblast proliferation and differentiation by evaluating alkaline phosphatase (ALP). Oxidative stress in bone cells results in the production of reactive oxygen species (ROS) from lipoxygenase and oxidase [9]. ROS can affect bone cells through decreased production of bone matrix protein (characterized by decreased ALP value) [10]. ALP is an identified biochemical marker of bone formation on the osteoblast plasma membrane reflecting osteoblastic activity on bone remodeling process [11] and plays an important role in osteoid formation and bone mineralization [12].

Materials and methods

Cell culture and reagents

Reagent chemicals, such as Alkaline Phosphatase Colorimetric Assay Kit, Acid Phosphatase Leukocyte Kit, and all other chemicals, were acquired by Sigma-Aldrich Co. (St Louis, MO, USA). All cell culture materials and solvents were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and analytical grade (J.T. Baker, USA). Mouse osteoblast-like cells (7F2) were obtained from Department of Biochemical Sciences & Technology, National Chiayi University, Taiwan, and refined in Dulbecco's Modified Eagle's Medium (DMEM). They were farther strengthened by 10% v/v Fetal Bovine Serum (FBS), 100 µg/mL streptomycin, and 100 units/mL penicillin. Cells were incubated in a dabbled incubator with 5% CO_2 at 37 °C.

Materials

The plants were collected in middle July 2018 in Baung Forest, Indonesia, and voucher samples were stored at Pharmacognosy Laboratory, Faculty of Pharmacy, Universitas Airlangga, Indonesia. The plants were identified by the Plant Conservation Institution, Purwodadi Botanical Garden.

Extraction

Fresh plants obtained from Baung Forest, Purwodadi, were cleaned and washed with clean running water, then dried under indirect sun to dry. After drying, the particle sizes were reduced by grinding until a powder was obtained. A total of 100–200 g of plant powders were extracted with 96% ethanol-aqueous (100 mL × 3) by maceration method. Each of 96% ethanol solution was evaporated using a rotary evaporator to get each of 96% ethanol extract (*E*) (Table 1). The potent extract was sequentially fractioned with hexane, ethyl acetate, butanol, and aqueous to provide hexane-soluble (EH), ethyl acetate-soluble (EE), butanol-soluble (EB), and aqueous-soluble (EA) fractions.

DPPH measurement

The antioxidant activity of 96% ethanol extracts was defined by di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) assay. The 0.25 mM DPPH solution was processed using DPPH solution in methanol. The 100 µg/mL of 96% ethanol extracts was mixed with 0.25 mM DPPH reagent in equal amounts (100 µL) in 96 well plates. Blank solution was the mixture of sample solvent (ethanol, 100 µL) and methanol (100 µL). DPPH reagent (100 µL) was mixed with methanol (100 µL) to serve as control. The reaction mixtures were shaken gently in the dark for 15–30 min at 25 °C. After the incubation, the absorbance was evaluated at 517 nm using a Tecan, infinite M200 microplate reader. The measurements were performed in triplicates. The DPPH radical scavenging was counted by equation [13, 14].

$$\text{DPPH radical scavenging effect} = \frac{(1 - \text{sample groups absorbance} - \text{blank absorbance})}{\text{Control group absorbance}} \times 100\%$$

Cell viability assay

The 7F2 cells were plated for cell growth studies at a density of 10^4 cells/well in 96-well plates. DMEM medium composing 100 units/mL penicillin, 10% FBS, and 100 µg/mL streptomycin was used to restore the cell. After 24 h, the E extract, EH, EE, EB, and EA fractions of *Elaeocarpus serratus* L. from Baung Forest were incubated at various concentrations for another 24 h at 37 °C. The cell supernatants were subsequently extracted, after 200 µL 3-(4,5-dimethylthiazol-2-yl)- and 100 µL of 2,5-diphenyltetrazolium bromide (MTT) reagent (100 µg/mL) were incubated during 4 h. Similarly, to dissolve the formazan crystals, 100 µL of dimethyl sulfoxide (DMSO) was added. The absorbance was ruminated at 570 nm by an enzyme-linked immunosorbent assay (ELISA) reader. All experiments were performed in triplicate, with the relative cell viability (%) declared as a portion relative to the unprocessed control cells [15, 16].

Differentiation of cellular alkaline phosphatase activity (ALP)

The 7F2 osteoblast-like cells were plated in 24-well plates at 10^4 in DMEM containing 5 mM β-glycerol phosphate (β-GP), 10% FBS, and 50 µg/mL of ascorbic acid (2GF medium) with or without E extract, EH, EE, EB, and EA fractions of *E. serratus* L. from Baung Forest for 4 and 7 days incubation period at 37 °C in a 5% CO_2 atmosphere. Phosphate buffered saline (PBS) was applied to clean the supernatants. After that, a percentage of the v/v triton solution was inserted and incubated for 10 min at 37 °C. After incubation, the cell lysates were examined for ALP by adding 200 µL of *p*-nitrophenyl phosphate (PNPP) and diethanolamine buffer into each well for a period of 30 min and at room temperature. The 50 µL/well stop solution was inserted to stop the reaction while ELISA reader at 405 nm was applied to measure the absorbance [15, 16].

Statistical analysis

The experiments were performed for three times using similar methods. It was then expressed as means ± standard deviations. The one-way ANOVA and LSD test were used to illustrate data analysis. The differences proved to be statistically significant at $p<0.05$.

Results

The effect of 96% extracts from Baung Forest plants on DPPH radical scavenging

During our project in order to discover antiosteoporotic delegates from natural sources [16–20], we screened 36 plants from Baung Forest on antioxidant by measuring DPPH scavenging. Oxidative stress produces a breakage of cellular owing to membranes structural change, lipid oxidation, and oxidation of nucleic acids and proteins. The breakage may expand to the organs and become systemic [21]. Many

Table 1: Baung forest plants collection and their DPPH radical scavenger effect (%).

No.	Name of plant	Indonesian name	Family name	Part of plant	*E* (% yields)	DPPH at 100 µg/mL, %
1	*Ixora nigricans*	Jejarum	Rubiaceae	Leaves	7.52	28.06 ± 2.19
2	*Brucea javanica*	Buah makasar	Simaraubaceae	Leaves	7.87	17.34 ± 5.56
3	*Mitrephora polypyrena*	Janglot, kalak	Annonaceae	Leaves	6.14	62.47 ± 1.92
4	*Hypoestes phyllostachya*	Polkadot	Acanthaceae	Leaves	8.76	19.60 ± 7.60
5	*Eranthemum nervosum*	–	Acanthaceae	Aerial part	8.07	22.22 ± 1.65
6	*Protium javanicum*	Trenggulum	Burseraceae	Aerial part	8.88	67.86 ± 3.30
7	*Urena lobata*	Pulutan	Malvaceae	Leaves	4.68	37.46 ± 8.24
8	*Blumea lacera*	Sembung kuwuk	Asteraceae	Leaves	11.66	11.87 ± 7.95
9	*Allophylus serratus*	–	Sapindaceae	Leaves	6.67	50.15 ± 5.61
10	*Melicope latifolia*	Parijoto	Rutaceae	Leaves	14.61	46.35 ± 2.42
11	*Plumbago zaelanica*	Daun encok	Plumbaginaceae	Leaves	4.46	29.73 ± 1.91
12	*Parameria leivigata*	Kayu rapet	Apocynaceae	Leaves	7.49	26.90 ± 3.85
13	*Elaeocarpus serratus*	Genitri	Elaeocarpaceae	Leaves	12.32	82.17 ± 2.95
14	*Reulia tuberosa*	Pletekan	Acanthaceae	Leaves	7.45	36.39 ± 5.72
15	*Dracaena elliptica*	Drakaena	Asparagaceae	Leaves	9.85	65.71 ± 3.30
16	*Garuga floribunda*	Kilangit	Burseraceae	Leaves	8.36	70.95 ± 3.37
17	*Sida acuta*	Sidaguri	Malvaceae	Aerial part	6.58	13.59 ± 4.82
18	*Plumeria acuatifolia*	Kemboja	Apocynaceae	Leaves	7.52	47.66 ± 9.66
19	*Memecylon myrsinoides*	Baho	Melastomataceae	Leaves	7.09	81.02 ± 1.17
20	*Solanum torvum*	Takokak	Solanaceae	Leaves	5.40	36.01 ± 4.88
21	*Solanum verbascifolium*	Terong teter	Solanaceae	Leaves	7.27	30.91 ± 5.14
22	*Lantana camara*	Saliara	Verbenaceae	Aerial part	6.82	15.45 ± 4.65
23	*Polyscias nodosa*	Tirotasi	Araliaceae	Leaves	10.70	–
24	*Harrisonia perforata*	Rui	Rutaceae	Aerial part	8.62	49.45 ± 4.18
25	*Hibiscus surattensis*	Waru	Malvaceae	Leaves	8.58	75.38 ± 1.92
26	*Lantana camara*	Saliara	Verbenaceae	Flos	11.29	18.94 ± 4.69
27	*Melanolepis multiglandulosa*	Daun kapur	Euphorbiaceae	Leaves	6.72	13.52 ± 3.72
28	*Rauvolfia tetraphylla*	Pule pandak	Apocynaceae	Leaves	11.16	32.06 ± 3.33
29	*Gloriosa superba*	Kembang sungsang	Liliaceae	Leaves	9.26	14.68 ± 1.92
30	*Centrosema pubescens*	Centro	Fabaceae	Flos	4.96	–
31	*Centrosema pubescens*	Centro	Fabaceae	Aerial part	7.94	15.35 ± 2.93
32	*Voacanga glandiflora*	Kalantong	Apocynaceae	Leaves	9.20	24.32 ± 1.63
33	*Phaleria octandra*	Mut	Thymelaeaceae	Leaves	8.96	6.09 ± 1.25
34	*Melia azedarach*	Mindi kecil	Meliaceae	Leaves	5.52	16.94 ± 2.32
35	*Hypoestes phyllostachya*	Polkadot	Acanthaceae	Leaves	6.08	71.47 ± 3.55
36	*Aglaia lawii*	–	Meliaceae	Leaves	11.99	30.12 ± 1.11

ailments have been related to oxidative stress and inserting bone diseases (osteoporosis). Antioxidants reduce acceleration of bone damage thru encoragement of tumor necrosis factor alpha (TNFα) [22]. Based on the screening result, the 96% ethanol extract of *E. serratus* L. (13), *Memecylon myrsinoides* (19), *Hibiscus surattensis* (25), and *Hypoestes phyllostachya* (35) from Baung Forest showed high DPPH radical scavenging (82.17 ± 2.95, 81.02 ± 1.17, 75.38 ± 1.92 and 71.47 ± 3.55%, respectively) (Table 1). Therefore, the most potent plant as an antioxidant is *E. serratus* L. In Indonesia, the leaves of this plant are used traditionally to treat arthritis [23] and in India, it is used as Ayurveda of anti osteoporosis [24] and arthritis [25]. Then, the % DPPH radical scavenging toward this plant at different concentration were explored.

The effect of 96% ethanol extract of *E. serratus* L. leaves on DPPH radical scavenging

In this research, we analyzed the effects of *E* extract, EH, EE, EB, and EA fractions of *E. serratus* L. from Baung Forest leaves toward antioxidant related to bone turnover. Several researches reported on the pharmacological effects of plant extract (Elaeocarpaceae family) from several countries

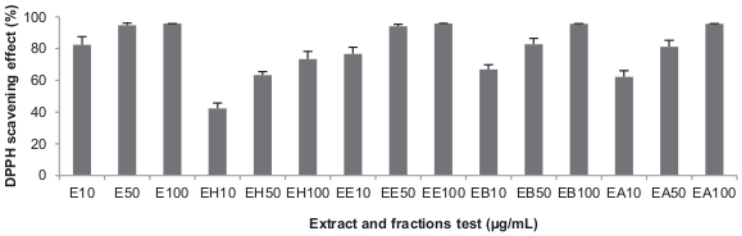

Figure 1: Antioxidant activity using DPPH method of 96% ethanol extract, hexane, ethyl acetate, butanol, and aqueous fractions of *E. serratus* leaves. The number of 10, 50, and 100 following extract (*E*) and fractions (EH, EE, EB, and EA) indicate their concentrations at 10, 50, and 100 µg/mL.

[23, 24, 26–29], but there have been no reports on 96% ethanol extract of *E. serratus* L. from Baung Forest, Indonesia.

DPPH is steady nitrogen that focuses on free radical that can receive hydrogen radical or electron to finish a steady diamagnetic molecule. DPPH radicals respond with appropriate reducing agents as a yield of which the electrons get couple off becoming the corresponding hydrazine. Thus, the antioxidant activity of *E* extract, EH, EE, EB, and EA fractions of *E. serratus* L. from Baung Forest with several concentrations (10, 50, and 100 µg/mL) was detected by DPPH scavenging assay in a range of concentration. Based on the result, the *E* extract, EH, EE, EB, and EA fractions had IC_{50} value of 23.27, 42.47, 19.93, 30.12, and 34.90, respectively (Figure 1).

The ALP stimulation effect of 7F2 osteoblasts of 96% ethanol of *E. serratus* L

The viability results of *E* extract, EH, EE, EB, and EA fractions of *E. serratus* L. from Baung Forest in 7F2 osteoblastic cell lines was carried out using MTT test. The viability cells of their extract and fractions increased in dose-related, in which they showed that high concentration of extract and fractions were not toxic (Figure 2) and elevated cellular uptake. Then, ALP experiments were proceed.

The ALP stimulation of 7F2 osteoblast cells using *E* extract, EH, EE, EB, and EA fractions of *E. serratus* L. from Baung Forest was incubated for 4 and 7 days. The results of samples on increasing ALP assay in the 7F2 osteoblasts againsted to the 2GF group on EH, EB, and EA fractions for 4 days (Figure 3). After 7 days, the EB, EA, and EH fractions stimulated their ALP activity to 148.56 ± 8.47, 144.58 ± 1.04, and 138.11 ± 9.72%, respectively (Figure 3).

Discussion

Geographically, the Baung Purwodadi forest area is located between 7°49′9″–7°47′23″ south latitude and 112°16′23″–112°17′17″ east longitude with the topography in general being bumpy to hilly, the altitude of this area ranges from between 200 and 501 masl, red–yellow mediterranean soil types and latosols, soil derived from old quarter rock with the main material in the form of metamorphic sediment, climate type D rainfall with a value of Q = 81.82%, the average annual amount of 2.654, 10 mm/year with an average number of rainy days of 141.05 days [30]. In the forest there are plant communities. Potential flora in the TWA Gunung Baung area, including *Brucea javanica*, *Urena lobata*, *Plumbago zaelanica*, *Parameria leivigata*, *Garuga floribunda*, *Plumeria acuatifolia*, *Lantana camara*, *Rauvolfia tetraphylla*, *Gloriosa superba*, *Melia azedarach* and others (Table 1). These plants are used by the local community for treatment such as lowering sugar levels, fever, inflammation, high blood pressure, treating stomach aches, relieving joint pain, headaches, worming, and urination.

The use of these plants as traditional medicine is only based on inheritance from ancestors without knowing the

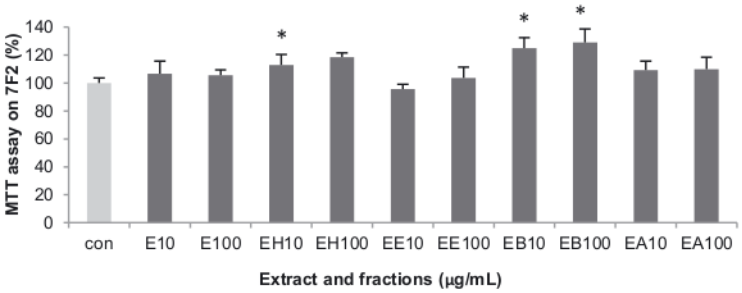

Figure 2: The MTT test of 96% ethanol extract, hexane, ethyl acetate, butanol, and aqueous fractions of *Elaeocarpus serratus* leaves on 7F2 osteoblast cells. The number of 10, 50, and 100 following extract (*E*) and fractions (EH, EE, EB, and EA) indicate their concentrations at 10, 50, and 100 µg/mL.

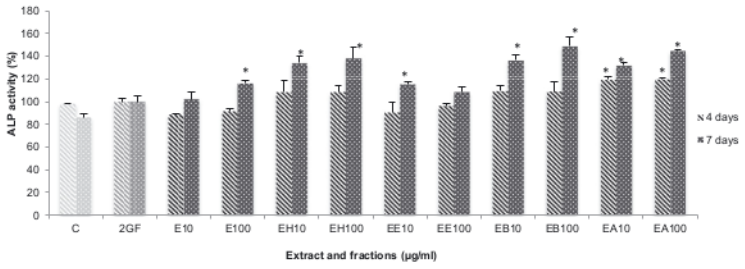

Figure 3: The ALP activity of 96% ethanol extract, hexane, ethyl acetate, butanol, and aqueous fractions of *Elaeocarpus serratus* leaves for 4 and 7 days incubation. The number of 10, 50, and 100 following extract (*E*) and fractions (EH, EE, EB, and EA) indicate their concentrations at 10, 50, and 100 µg/mL. The sign * means p<0.05–2GF.

chemical content that plays a role in treatment [31]. Therefore, to determine the exact chemical content for treatment, it is necessary to explore plants, especially forest plants that have a large enough potential. The initial screening was antioxidant potential because the assay is simple and easy for large quantities. Oxidative stress occurs as a result of overproduction of ROS which is not balanced, which can cause bone disruption. The altered redox state is also associated with the bone remodeling process which enables the continuous regeneration of bone through the coordinated action of bone cells. Changes in ROS and/or the antioxidant system involve in the pathogenesis of bone loss. ROS induces apoptosis (death) of osteoblasts and osteocytes, this encourages osteoclastogenesis and inhibits mineralization and osteogenesis [32]. Based on DPPH Radical Scavenging result on several plants in the Baung forest, *E. serratus* L. has the highest potential in trapping DPPH radical scavening (82.17 ± 2.95). Therefore, it continues the exploration of this plant to determine their ability to increase bone density.

Natural plants have performed a pivotal part in pharmaceutical drugs and dietary supplement developments for the therapy and precaution of ailment [33]. One of them is *E. serratus* L. from Baung Forest which belongs to the Elaeocarpaceae family. Traditionally, it is used to treat migraine, stress, anxiety, depression, lack of concentration, palpitation, nerve pain, epilepsy, asthma, hypertension, liver diseases [23], arthritis [34], Ayurveda of anti osteoporosis [24], and Ayurveda of osteoarthritis [25]. Several studies have shown that this plant is pharmacologically active and can be functioned as the treatment of arthritis [35], antimicrobial [36], anti inflammatory, analgesic, pesticide, nematoside, antioxidant [25], antibacterial, diarrhea, and dysentery [37]. The leaves contain flavonoids, carotenoids [34, 38], fatty acid [26], myricitrin, and mearnsetin derivatives [39]. Myricitrin has the greatest antioxidant activity in this plant [39]. It was also proved in this study that 96% ethanol extract of *E. serratus* L. leaves had a radical scavening DPPH value of 82.17 ± 2.95% (Table 1). This is the greatest value of its activity compared to other plant extracts from Baung Forest. Based on Figure 2, almost all fractions has the ability to trap free radical >50% at concentration of 10–100 µg/mL but the hexane fraction (EH) at 10 µg/mL cannot trap DPPH radicals by up to 50%. The greater percentage value of trapping, the better antioksidan activity in DPPH radical scavening [40]. Consequently, we explored this extract for bone formation activity.

Several studies have associated antioxidants with bone metabolism. Lower plasma antioxidants can be found in elderly women or women with osteoporosis. Oxidative stress in estrogen deficiency of postmenopausal osteoporosis has been linked to the activation of NADPH oxidase and/or alleviated synthesis of antioxidant enzymes and glutathione (GSH) levels [21, 26]. This antioxidant leads the acceleration of bone loss through activation of tumor necrosis factor alpha (TNFα) [22]. Converting in the redox state is also linked to the process of bone remodeling that permits continuous bone regeneration thru coordinated action of bone cells such as osteoblasts, osteocytes, and osteoclasts. Antioxidants directly contribute to activating osteoblast differentiation in bone formation and mineralization processes.

Based on the results, the 96% ethanol extract of *E. serratus* L. leaves had a strong antioxidant activity and also played a role in the activation of osteoblast differentiation which is directly related to bone formation. Osteoblast differentiation is characterized by measuring levels of ALP. ALP is an important enzyme that is a useful biochemical marker of bone formation [41]. This enzyme plays a role in osteoid formation and mineralization. So that the ALP enzyme and bone mineralization have a significant correlation and become a biochemical marker [42]. Bone growth and healing during bone fracture cause high ALP enzymes in bones. However, if the ALP enzyme appears in excess, it can be an indicator of osteosarcoma to bone metastases [43]. The 96% ethanol extract of *E. serratus* L. leaves stimulated ALP activity in dose of dependent manner (116% of 100 µg/mL). Among the fractions, EB had the strongest ALP activity (148.56 ± 8.47%). It is a potential fraction for activation of bone formation. Ethanol extract from this plant contains fatty acid ester derivatives such as

n-dotriacontanol (10.70%), *n*-octadecanol (10.08%), docosanoic acid, 1,2,3-propanetriyl ester (9.07%), *n*-hexadecene (8.52%), bis-(3,5,5-trimethylhexyl) ether (6.30%), ethanone, 1-cyclopentyl- (4.81%), cyclohexane, ethyl- (4.05%), and minor components were hexadecanoic acid methyl ester (0.80%), ricinoleic acid (0.77%), citronellyl isobutyrate (0.69%), and farnesol (0.51%) [26]. Fatty acid has a role in increasing bone formation by stimulated β catenin activity in osteoblast and resulting in increased in osteoblastogenesis [44, 45]. The mechanisms of fatty acid are complex and involve protectins and resolvins, prostaglandins, growth elements, cytokines, and few other molecular signaling routes [45]. This plant also contains carotenoids [38], that have a encourage effect on osteoblastic bone formation *in vitro*, therefrom escalating bone mass. This effects the gene expression of various proteins associated to bone formation [45]. Thus, the 96% ethanol extract of *E. serratus* L. leaves has potential effect to maintain bone health and decrease bone loss.

Conclusions

The extract and fractions of *E. serratus* L. can successfully inhibit DPPH radical scavenging value and increase ALP activities as markers of osteoblast functions.

Acknowledgments: The authors are grateful for access given by Department of Biochemical Science and Technology, National Chiayi University, Chiayi, Taiwan, Republic of China.

Research funding: The research was supported by Penelitian Dasar (basic research) grant from Ministry of Research, Technology and Higher Education, republic of Indonesia with the contract no. 4/E1/KP.PTNBH/2019 and 637/UN3.14/LT/2019.

Author contributions: We declared that this work was done by Retno Widyowati (RW), Neny Purwitasari (NP), Rice Disi Oktarina (RDO), Wiwied Ekasari (WE), Saarah Khairunnisa (SK) and Hsin-I Chang (HC). NP collected the antioxidant data, WE analyzed the antioxidant data, SK collected the ALP data, RDO analyzed the ALP data, RW designed the study and wrote the manuscript, HC designed the study and analyzed the ALP data. All authors had read and approved the manuscript.

Competing interests: No conflict of interest was associated with this work.

Informed consent: Not applicable.

Ethical approval: Not applicable.

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