ORIGINAL ARTICLE

Sasaladaan (*Peperomia pellucida* [L.] Kunth.) Extracts Improve Trabecular Bone Microarchitecture in Ovariectomy-Induced Osteoporotic Rats

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Abstract

Introduction: Several studies have been conducted to prove the potential of Sasaladaan (*Peperomia pellucida* [L.] Kunth.) as an antiosteoporosis, but this still needs to be proven directly on bone tissue. Therefore, this study aims to quantitatively measure the parameters of advanced microarchitecture on bone samples of test animals and completed using an antioxidant activity assay to assess its mechanisms. **Materials and Methods:** Bone tests were conducted using microcomputed tomography scans and the antioxidant test was conducted using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) methods. **Results:** The ethanol extract of Sasaladaan gave marked improvement to bone microarchitecture in the trabecular number, the structure model index, as well as the trabecular pattern factor parameters. In the antioxidant assay, the ethanol extract showed strong antioxidant activity (IC_{50} = 73.37 ± 4.32 ppm) when tested with DPPH reagent and showed moderate activity (IC_{50} = 128.99 ± 11.93 ppm) using the ABTS method. **Conclusion:** The effect of Sasaladaan on bone microarchitecture repair is demonstrated using the ethanol extract at a dose of 100 mg/kg body weight of rat. The antioxidant activity that was present may support the substance's antiosteoporotic activity.

Key words: Antioxidants, bone, osteoporosis, *Peperomia pellucida*, trabecular

INTRODUCTION

Osteoporosis is a disease that affects the bones and is characterized by reduced bone mass and changes in bone tissue microarchitecture. This results in decreased bone strength and increased bone fragility, easily causing bone fractures.[1] Empirical treatments for osteoporosis can be found throughout the world, including the use of the Sasaladaan (*Peperomia pellucida* [L.] Kunth.) plant.[2,3]

The Sasaladaan plant, member of the Piperaceae family, is now widely cultivated in many parts of the world.[4,5] This plant is local vegetables in Thailand.[6] The young shoots and stems are blanched and eaten with *nam phrik* or *laab*. Filipinos eat it in green salad.[7] In Indonesia, this plant is consumed as "lalapan," tea, and used as a medicinal plant.[8,9] In Cameroon, this plant has been empirically used for treating fractures.[2,3] The parts of the plant or entire plant or have also been used as decoctions or macerates to be administered orally or applied as a paste to the site of the fracture.[3]

This plant has been reported to have a large variety of nutritional content, including proteins, lipids, carbohydrates, and minerals such as potassium, calcium, iron, sodium, zinc, copper, magnesium, and phosphorus.[10,11] The average potassium and calcium contents of the plant were

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6977 mg/100 g and 483 mg/100 g dry weight, respectively. In addition, the calorie content of 100 g of the dry plant is 258 kcal.[10] Due to its high nutritional content, the Sasaladaan plant is used as a source of energy and minerals that are especially beneficial for bones.

Many researchers have proven that it had various pharmacological activities including antiosteoporoticactivities.[5] The efficacy of Sasaladaan as an antiosteoporosis agent is based on a study by Xu *et al*., who discovered agonist activity of 17β-estradiol (a type of estrogen) of an active compound 7,8-trans-8,8'-trans-7', 8'-cis-7,7'-bis (5-methoxy-3,4-methylenedioxyphenyl)-8methyl acetoxy-8'-hydroxy methyl tetrahydrofuran.[12] Estrogen, as well as the class of compounds known as phytoestrogens, plays a major role in the prevention and treatment of osteoporosis.[13] The estrogenic activity of the ethanol extract of Sasaladaan is reinforced by *in vitro* and *in vivo* research investigating the biochemical profile of rats with induced ovariectomy.[14,15] However, the direct efficacy of the plant extract on bone repair, as well as its mechanisms of action, still needs to be proven. This plant is an interesting object to be tested not only due to its potency on osteoporosis treatment as described but also because it is easy to find and cost-efficient.

The purpose of this study is to measure the quantitative effects on bone microarchitecture and the antioxidant activity of the Sasaladaan plant. Sasaladaan is expected to be a new and alternative treatment for osteoporosis with a high potency.

MATERIALS AND METHODS

Materials

Sasaladaan herb, aquadest, aquabidest, ethanol 96%, ethyl acetate, n-hexane, formalin, hematoxylin, eosin, HCl, sulfonic acid, acid acetate anhydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) reagent, dimethyl sulfoxide (DMSO), and methanol were used.

Rat Femur Bones

Rat femur bones were obtained from a previous study.[15] The study followed approval protocol for animal handling from the Institutional Animal Ethics Committee of School of Pharmacy, Institut Teknologi Bandung with ethics number 04/KEPHP-ITB/03-2015. The bones were stored in the saline-soaked bandages at −20°C for 4 months. Through physical examination and first microcomputed tomography (μCT) scan results, the bones were still in good condition for further testing. In the previous study, the animals underwent sham or ovariectomy (OVX) surgery with a double dorsolateral approach. Rats in the sham control group were operated in the same manner as the OVX group but without excision of the ovaries. 1 week after the operation, the animals were divided into seven groups. Sham and ovariectomized control group received the vehicle (0.3% CMC-Na). The remaining OVX rats were treated with a standard drug (ethinyl estradiol) at a dose of 4.5 μg/kg, Sasaladaan juice at a dose of 50 or 100 mg/kg, and Sasaladaan herb ethanolic extract at a dose of 50 or 100 mg/kg. The treatments were given daily for 6 weeks.

Extraction Process

Samples of the Sasaladaan plant were obtained from Cagak and Ciater, Subang, West Java, in March–April 2016. The plant was authenticated at the Herbarium Bandungense, Bandung Institute of Technology, Indonesia. 500 g of the dry plant were extracted using, in order, n-hexane, ethyl acetate, and 96% ethanol using the maceration method for 3 × 24 h. In addition, a water extract was also prepared using the same method.

μCT

Rat femur bones were tested with Bruker SkyScan1173® μCT scanner and with high resolution.[16] Specimens are aligned with the vertical axis of the scanner, with no medium. In this research, 45 kV voltage, 80 μA current, 700 ms exposure time, and 0.2° rotation step were used. Frame averaging and random movement with the value of 10 and a 1.0 mm aluminum filter are also used in the scanning progress. A set of projection images (±1750 images) of the specimen was successfully obtained with resolution of 12.11 μm per pixel with the dimension of 2240 × 2240 pixels. The projection images were reconstructed using NRecon® with GPUReconServer reconstruction kernel. The image qualities were checked further with the DataViewer® and image analysis of the reconstructed gray scale images was performed using the CTAn®. As many as, 250 slices of the trabecular bone were analyzed for the region of the distal metaphysical, which is located 0.75 mm below the growth plate. For the cortical bone, analysis was carried out on 20 slices of the diaphysis area, 3.07 mm distal from the growth plate. The results were then compared to that of the analysis of the mid-diaphysis area with the same number of slices. The trabecular parameters measured were trabecular number (Tb.N), structure model index (SMI), and trabecular pattern factor (Tb.Pf). The data were then analyzed using the IBM SPSS Statistics software version 20®. Qualitative comparison of the thickness of the distal metaphysis and mid-diaphysis of the rat cortical bones was performed by visual assessment.

Histology

After μCT scans, the femur bones were then prepared for histologic examination using standard methods. A 10% HCl solution was used to decalcify the femur bones for about 3 h. Staining was performed using hematoxylin and eosin. The bone microarchitecture profiles of the preparations were

observed using a binocular microscope (Olympus®) at ×40 magnification and the Optilab Viewer® application.

Antioxidant Activity Test

Antioxidant activity of different extract of Sasaladaan herb was measured by DPPH and ABTS. In brief, 50 ppm solution of DPPH in methanol as solvent was prepared. This solution (200 µL) was added to 50 µL of different samples in methanol at different concentration (100, 50, 25, and 12.5 µg/mL) on 96 microwell plate. The mixture was shaken vigorously for 10 s and allowed to stand at room temperature for 30 min. Then, absorbance was measured at 517 nm using microplate reader INFINITE 200 PRO.

In the ABTS method, the radical cation was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (1/1, v/v) and leaving the mixture for 16–18 h until the reaction was complete and the absorbance was stable. The ABTS solution was diluted with DMSO to an absorbance of 0.700 ± 0.05 at 734 nm for measurements. The photometric assay was conducted on 180 µL of ABTS solution and 20 uL of tested samples (100, 50, 25, and 12.5 µg/mL) on 96 microwell plate and shaken for 10 s. Measurements were taken immediately at 734 nm after 7 min.

The percent antioxidant activity was calculated using the following equation:

(%) antioxidant activity = Ac-At/Ac × 100

Where, At was the absorbance of samples and Ac the absorbance of methanolic DPPH solution (DPPH method) or absorbance of ABTS (ABTS method).

Reference standard compound being used was ascorbic acid and α-tocopherol. The IC_{50} value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical or radical cation, was calculated using ln dose-probit percentage antioxidant activity curve. The lower absorbance of the reaction mixture indicated higher antioxidant activity.

Statistical Analysis

SPSS package version 20.0 for Windows was used for the analysis. Normally, distributed data were analyzed using one-way analysis of variance with Tukey test while Kruskal–Wallis test using Bonferroni adjustment was used to analyzed the non-normally distributed data. All values were expressed as mean ± SD. The parameters value was considered statistically significant at $P < 0.05$.

RESULTS

Bone Microarchitecture Profile by µCT Scans

The qualitative and quantitative results of the bone microarchitecture examinations using µCT scans are shown in Figure 1 and Table 1. It can be observed from the scan results that the rats in the ovariectomy group had the worst trabecular bone profile, and that administration of estradiol significantly improved the profile. Furthermore, the ethanol extracts were able to repair the bones better than Sasaladaan juice, with higher effects at increasing doses. However,

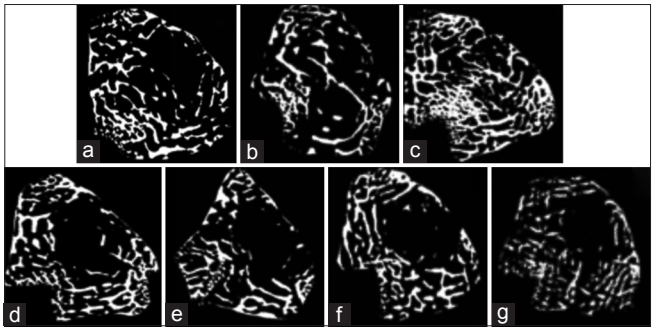

Figure 1: Trabecular bone microarchitecture profiles by Bruker SkyScan1173® microcomputed tomography scanner with high resolution for the normal (a), ovariectomy (b), estradiol (c), juice 50 mg/kg bw (d), juice 100 mg/kg bw (e), ethanol extract 50 mg/kg bw (f), ethanol extract 100 mg/kg bw (g). The white areas of the image are areas of high bone density (mineralized bone), whereas the dark areas are areas of low density (cavities or air)

Table 1: Comparison of trabecular bone microarchitecture parameter values of the treatment groups by Bruker SkyScan1173® µCT scanner with high resolution in the Tb.N., the SMI, as well as the Tb.Pf parameters

Type of groups	Tb.N. (1/mm)	SMI	Tb.Pf. (1/mm)
Normal	12.95[a]±1.28	1.60±0.33	9.77±0.29
Ovariectomy	10.36[b]±0.39	2.00±0.11	11.41±1.76
Estradiol	11.89±0.47	1.68±0.44	10.23±1.34
Juice 50 mg/kg bb	11.39±1.17	1.89±0.11	10.81±0.33
Juice 100 mg/kg bb	11.38±0.08	1.87±0.11	9.36±1.73
Ethanol extract 50 mg/kg bw	11.37±0.26	1.86±0.04	10.32±0.83
Ethanol extract 100 mg/kg bw	11.60±0.60	1.84±0.22	9.24±1.25

The value was performed in mean±SD. [a–b]Mean with the different letters in each column are significantly different, Mct: Microcomputed tomography, SMI: Structure model index, Tb.N.: Trabecular number, Tb.Pf: Trabecular pattern factor

estradiol, a conventional drug for osteoporosis, restored the bone condition to a normal state.

Distal Metaphysis and Mid-diaphysis Cortical Area Thickness

The thickness of the distal metaphysis and mid-diaphysis cortical bone areas of the rats in the normal group was observed by both μCT scans and histologic examination [Figures 2 and 3]. The results show that, in general, the mid-diaphysis area is thicker than the distal metaphysical region. This was also observed in the results of the histologic examination.

Antioxidant Activity Assay

The Sasaladaan extracts were tested for their antioxidant activity using the DPPH and ABTS methods, and the results are shown in Table 2. Vitamin C and E, as the standard antioxidants used, resulting in very low IC_{50} values, show very strong antioxidant activity. In both DPPH and ABTS methods, the ethanol extract exhibited strong and moderate antioxidant activity, relatively. Despite, its activity was not as strong as the activity of both standard antioxidants.

DISCUSSION

A bone microarchitecture profile is an important parameter to be examined when investigating how a drug is able to improve the condition of the bone. Results of the μCT scan showed that the Sasaladaan ethanol extracts were able to improve the bone microarchitecture in osteoporosis-induced rats better than Sasaladaan juice. The relative effect of the ethanol extracts compared to estradiol was 0.66 for Tb.N., 0.44 for SMI, and 0.92 for Tb.Pf at dose of 50 mg/kg and 0.81 for Tb.N., 0.5 for SMI, and 1.83 for Tb.Pf at dose of 100 mg/kg.

These results are in line with the results of a previous study, in which *P. pellucida* ethanol extracts decreased serum alkaline phosphatase levels and urinary excretion of calcium more than *P. pellucida* juice.[15] However, the effect of the ethanol extract did not differ significantly compared to that

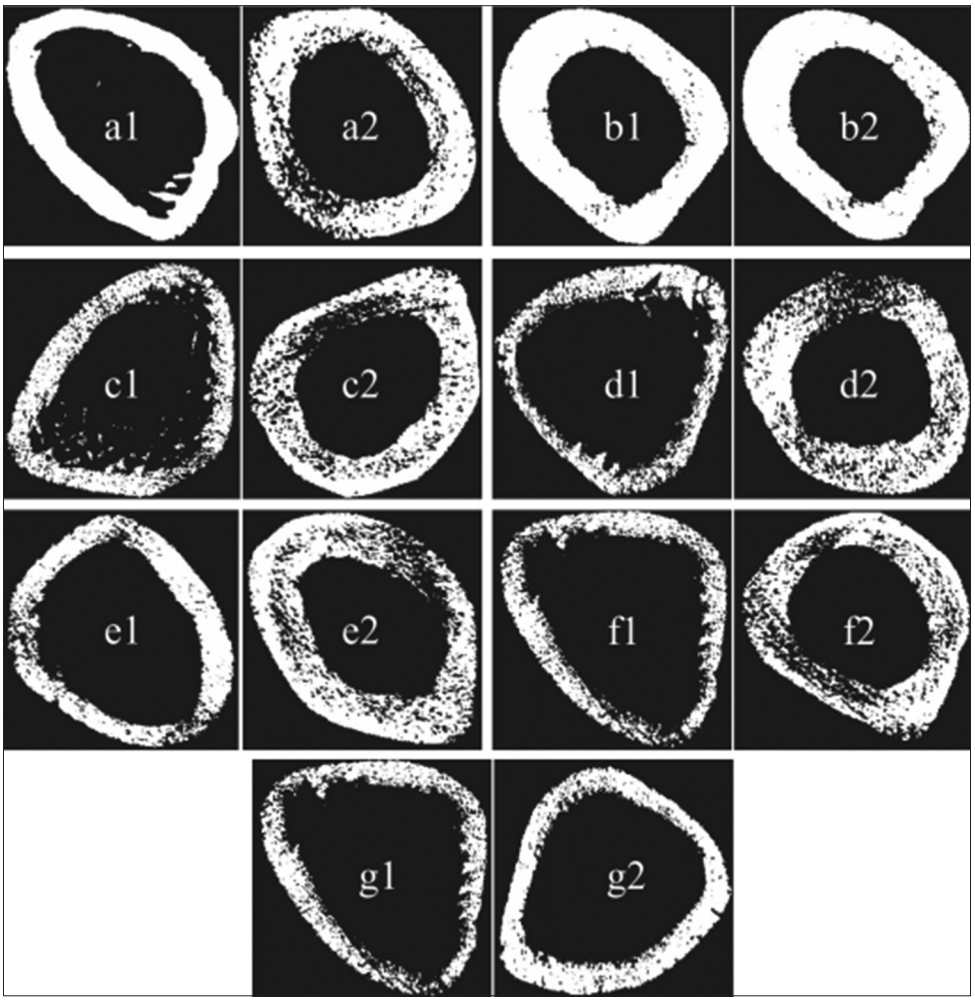

Figure 2: Comparison of cortical bone thickness by microcomputed tomography scans. On the images, the distal diaphysis (1) and mid-diaphysis (2), [a]normal, [b]ovariectomy, [c]estradiol, [d]juice 50 mg/kg, [e]juice 100 mg/kg, [f]the ethanol extract of 50 mg/kg, and [g]ethanol extract 100 mg/kg

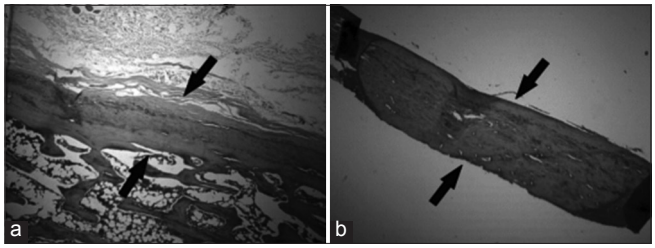

Figure 3: Comparison of cortical bone thickness by histologic examination with hematoxylin and eosin staining at ×40 magnification. The images are from the normal group. The thickness of the cortical bone (the area indicated by arrows) in the mid-diaphysis area (b) is bigger than the cortical bone in the distal diaphysis area (a)

Table 2: Antioxidant activity (IC_{50}) from the extract of *Peperomia pellucida* (L.) Kunth. herb using DPPH and ABTS methods

Sample type	IC_{50} (ppm)	
	DPPH	ABTS
Vitamin C	4.81[a]±0.13	5.39[a]±0.55
Vitamin E	14.13±4.61	8.36±0.44
N-hexane extract	>1.000	>1.000[b]
Ethyl acetate extract	526.89±74.51	157.79±13.51
Ethanol 96% extract	73.37±4.34	128.99±11.93
Water extract	>1.000[b]	255.12±20.33

The IC_{50} (ppm) was performed in mean±SD. [a–b]Mean with the different letters in each column are significantly different. DPPH: 2,2-Diphenyl-1-picrylhydrazyl, ABTS: 2, 2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid

of the juice. This may be due to the fact that the juice has been shown to increase serum calcium levels. This effect was better than the effect of the ethanol extract.[15] Increased levels of calcium improve the formation of bone matrix, which would certainly have an impact on bone microarchitecture improvements.

Estradiol, a form of estrogen, was used as the standard of antiosteoporosis medication in this study. While it is used to repair the bone matrix in ovariectomy operations, it is not able to restore normal bone conditions. However, it showed strong effect to restore bone condition. The results of this study show that administration of Sasaladaan ethanol extract at a dose of 100 mg/kg body weight improved the rats' bone microarchitecture but not as good as estradiol except in Tb.pf parameter.

Observation parameters of bone microarchitecture are not only limited to the observation of the trabecular bones but can also include observations of the cortical bones. µCT scans can be used to observe the thickness and porosity of cortical bones, which represent the quality of the bone. Results of analyses using this method are dependent on the selection of the bone area to scan. So far, there are differences in opinions regarding the exact area to analyze the parameters of the cortical bone. Some suggested the analysis conducted on the diaphysis area which is 3.07 mm from the distal growth plate and others suggest the analysis carried out in the area of mid-diaphysis.[17,18]

For accurate analysis of cortical bones to be performed, the bone area with the optimum proportion of cortical bone and minimal trabecular bone should be used. Based on qualitative observations, the mid-diaphysis area was thicker than the distal metaphyseal area [Figure 2]. In addition, trabecular bones were observed in the distal metaphyseal region, but none were observed in the mid-diaphysis area.

The data obtained were consistent with the profile of cortical bone in rabbits. Young rabbit femoral cortical thickness at the mid-diaphysis area was larger than in the distal area.[19] Parameters of cortical bone thickness are very useful in predicting bone strength and resistance to fractures.[20] Therefore, it can be said that the rat femur distal metaphyseal area is more susceptible to fractures than the mid-diaphysis area. Furthermore, the distal metaphyseal area is more appropriate to be used in a mouse osteoporosis model.

The µCT scans have shown that the ethanol extract of Sasaladaan has antiosteoporosis activity in an ovariectomy-induced rat animal model that mimics the pathological conditions of osteoporosis due to estrogen deficiency. Other researchers have used the same induction model to examine the antiosteoporosis effects of plants of the same Piperaceae family, namely *Piper sarmentosum*.[21] Although the study measured different bone parameters, *P. sarmentosum* extracts demonstrated the ability to improve the condition of bone fractures by decreasing the mean axial callus volume and median callus scores.

Antiosteoporosis activity is typically strengthened by a variety of other activities such as anti-inflammatory, analgesic, and antioxidant activities. Antioxidant activity correlated with antiosteoporosis activity due to the fact that severe oxidative stress has been implicated in many diseases including osteoporosis.[22] Indeed, the presence of strong antioxidants such as Vitamin C and Vitamin E, which provide protection against reactive oxygen species, demonstrated a positive effect on the physiological conditions of the bone. Vitamin C can increase colagenogenesis, while Vitamin E deficiency can disrupt transport through intestinal calcium, resulting in lower bone density.[23]

Out of the extract of Sasaladaan extract, the best antioxidant activity was shown by the ethanol extract, indicating that the antioxidant activity of Sasaladaan herb is contributed mainly by semi-polar and polar compounds. These results differ from those of previous studies.[24] In the study, ethyl acetate extract of Sasaladaan, which contains the most semi-polar components, had the highest total phenolic content and a higher antioxidant activity compared to the methanol and butanol extracts, with an IC_{50} value of 74.0 ± 0.52 µg/mL.

In this study, the antiosteoporosis of the ethanol extract and antioxidant activities of four types of Sasaladaan extract with different polarity were studied. Compared to *P. sarmentosum,* which has been proven to have the same activities, and the standard antioxidants Vitamins C and E,[25] the antioxidant activity of Sasaladaan is much lower. This suggests that the antioxidant activity of Sasaladaan plays a minor role in its antiosteoporosis mechanism.

Based on its activities and its nutritional composition, it can be concluded that Sasaladaan has the potential to be functional food, especially for improving bone health. The LD_{50} of the Sasaladaan ethanol extract was found to be above 5000 mg/kg in mice, and thus, the extract is categorized as non-toxic. Considering its extract yields and moisture contents, approximately 324 g of fresh *P. pellucida* plants every serves 2 times a day can be consumed to maintain healthy bones. In the next research, authors will further investigate the potential of *P. pellucida* (L.) Kunth. and its chemical compounds as antiosteoporotic.

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