Antiosteoporotic Activity of *Pandanus odoratissimus* Seed Extract in Ovariectomized Rats

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Abstract

Osteoporosis often called "silent diseases" of aging because bone loss occurs without symptoms, untuil microarchitectural deterioration and bone fractures occurs. The common therapy to prevent the estrogen degrading condition is Hormon Replacement Therapy (HRT). However, HRT possessed various risks. Recent studies suggest the phytoestrogens may exert a protective effect against osteoporosis. This study examined whether treatment with phytoestrogen extract from *Pandanus odoratissimus* seed (PS) exerted a preventive effect on estrogen deficiency induced osteoporosis. Three months old female Sprague Dawley rats were randomly assigned into either a sham operated group or one of five variectomy (OVX) groups, Negative control, positive control (with Allendronate) and OVX with PS extract. Rats began receiving treatment 20 days after the OVX and receiving treatment for 28 days after OVX. The results showed that PS (900mg/kb bw) treatment prevented decrease in trabecular density while also preventing an increase in trabecular separation and PS 900 mg/kg bw had the smallest proportion of osteoclast to osteoblast, compare to other treatment. The result of this study demonstrated that the potential effects of *Pandanus odoratissimus* seed extract (PS 900mg/kg bw) as herbal drug. Therefore it is suggested that *Pandanus odoratissimus* seed extract might be a potential alternative therapy for treating postmenopausal osteoporosis.

Keywords
osteoporosis, ovariectomized-induced model, trabecular density, herbal drug

1. Introduction

Osteoporosis often called "silent diseases" of aging because bone loss occurs without symptoms, until microarchitectural deterioration and bone fractures occurs (Deyhim et al, 2006). Osteoporosis is defined by the World Health Organization (WHO) as a 'progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture' (Liu et al. 2019). Osteoporosis affects 200 million women worldwide, and the probability of women over 50 affected by an osteoporotic fracture has been estimated to approach one third (Kanis, 2007). It threatens the health of middle-aged and the aged, especially postmenopausal women (Brown, 2017). Approximately half women over the age of 50 years are expected to suffer an osteoporosis -related fracture over their remaining lifetime (Xu et al 2016).

Post menopausal women are at high risk of developing osteoporosis because of the significant alterations in bone metabolism associated with estrogen deficiency (Andersen and Kristensen 2015). The rapid loss and higher bone fragility that take place during menopause lead to an increased incidence of spine, hip and wrist fractures in post menopausal women (Wang et al 2014). People who suffer from osteoporosis commonly encounter fragility fracture thus create a tremendous burden on the society.

Osteoporosis frequently occurs in the elderly and in menopausal woman, and the most wildly used anti-osteoporosis drugs are anti-resorptive bisphosphonates that inhibit the activity of osteoclasts. The current treatments for osteoporosis include anti-absorption therapy, such as bisphosphonate, calcitonin, and selective estrogen receptor modulator (Jones et al. 2005). But these medications for long-term use cause severe adverse effects like hypercalciuria, hypercalcemia, musculoskeletal pain, osteonecrosis of the jaw, breast tenderness, thromboembolic events, and increased risk of endometrial and breast cancer. These adverse effects of anti-resorptive drugs warrant development of anabolic agents to treat osteoporosis. Unlike anti-resorptive agents,

anabolic agents stimulate proliferation or differentiation of osteoblasts, and consequently, improve both quality and quantity of bone (Khan and Khan, 2006).

Postmenopausal osteoporosis which occurs in aging women is usually associated with estrogen deficiency (Riggs et al, 2002). The absence of ovarian hormone will result in accelerated bone resorption by osteoclasts and reduced bone formation by osteoblasts (Chavan et al. 2007). Oxidative stress has been implicated in the pathogenesis of osteoporosis as evidenced by numerous in vitro and in vivo studies (Muthusami et al. 2005). Oxidative stress occurs when the body's antioxidant defense is unable to overcome the cellular damage caused by free radical molecules (Jagger et al, 2005). Supplementation with antioxidants like vitamins C (Khassaf et al. 2003) and E (Ahmad et al. 2005) has been shown to prevent bone loss in osteoporosis.

Antioxidant systems play important roles in the development of osteoporosis. Evolving evidence suggests that ROS are involved in osteogenesis, including bone formation and resorption, which are associated with the aging process and may lead to osteoporosis (Kong et al. 1999). The studies have found that many medicinal plants have the potential to prevent and treat osteoporosis. Some bioactive compound seem to have important implications beyond the inhibition of bone resorption through supressing osteoclast activation. Probably, their positive actions of these compounds are mainly due to their antioxidant characteristics.

Antioxidant rich plant may represent a possible approach for slowing down age-related bone mass reduction and enhancing bone remodeling. *Pandanus odoratissimus* seed extract exhibit significant antioxidant activity, IC = 31.25 µg/ml, which was found to be more potent than those of reference antioxidant, ascorbic acid with IC= 73,61 µg/ml (Rahayu et al. 2020). The aim of the present study was to evaluate the possible beneficial effect of *Pandanus odoratissimus* seed extract on bone loss in the ovariectomized rat model of osteoporosis.

2. Materials and Methods

2.1 Plant material

The *P. odoratissimus* seeds were collected from Calang village, Aceh Jaya district, Aceh Province. The plant fruits from which the seeds were collected were mature, shown by the orange color of its exoderm. These seeds were shade dried, and mechanically grinded to a powder form.

2.2 Preparation of Methanolic Extract of *Pandanus odoratissimus* (POE)

Seed About 1000 g of the seed powder were subjected to cold maceration using 98% methanol for 15 days, while changing the solvent every 5 days. The collected solvent were filtered with whatman paper and then evaporated at 40 ^{o}C under low pressure till a semisolid residue was obtained.

2.3 Preliminary Phytochemical Screening

The qualitative phytochemical evaluation of the methanolic extracts was conducted to determine the presence or absence of flavonoids through Shinoda test, sterols through Libermann Buchard test, phenols through ferric chloride test, alkaloids through Dragendorff test, and saponins through saponification test, as described by Dee et al (2010).

2.4 Animals

Adult female Sprague Dawley rats weighing 180-200 g were obtained from the animal house of Food and Drug Research Agency, Jakarta, Indonesia. Rats were housed in polypropylene cages in air-conditioned room at a temperature of 23 ± 2 °C and under natural day and night cycle. They were fed standard pellets and drinking water ad libitum. The rats were kept for a week before the commencement of the experiment for acclimatization. The experimental protocol was approved by the Comission of Health Research Ethics of Universitas Pembangunan Nasional Veteran, Jakarta, Indonesia. All the experimental procedures were carried out in accordance with international guidelines for the care and use of laboratory animals.

2.5 Animals and group

Thirtysix healthy female Sprague Dawley rats, 12 weeks old and with body weight 200 g. After 1 week of adaptive feeding, the rats were randomly divided into 6 groups, with 6 rats in each group, as follows: sham operation group (control group, normal rats), osteoporosis group, 3 groups of rats with induced osteoporosis and treated with different concentrations of POE (300 mg/kg body weight [b.w.], POE 600 mg/kg b.w., and POE 900 mg/kg b.w.) and a group of rats with induced osteoporosis and treated with alendronate [ALD] (0.18 mg/kg b.w., every day). POE and ALD were administered orally, and sham and osteoporosis group received the same amount of vehicle buffer.

2.6 Osteoporosis model and treatments
Osteoporosis was induced by bilateral ovariectomy; the rats were anesthetized by intraperitoneal injection (IP) ketamine and xylazine. The abdominal skin and muscles were opened, the ovarian arteries were ligated in the uterus, and bilateral ovariectomy was performed. The small adipose tissue around the ovary was resected in the control group. The drugs were administered orally with volume of 0.5 ml; POE and ALD administered daily. The control and osteoporosis groups were treated with physiological saline. The treatments lasted for 4 weeks.

2.7 Body Weight, Uterine Weight
Body weight was measured every day basis to monitor health and measure weight gain. At the end of the study, the rats were anesthetized. Uterine weights were determined after removal of the uterus.

2.8 Bone histomorphometric evaluation
The proximal tibial was employed to investigate bone histomorphometry as previously reported (Tantikanlayaporn et al, 2013). Left tibia was collected, removed of the adhering tissues, fixation in 10% BNF for 48 hours. Then dehydrated through a graded series of alcohol 70%, 95% and 100%, decalcification with NA2EDTA, embedded in paraffin block. These embedded tibia block were longitudinally sections by microtome at the thickness of 5 μm . The 5 μm sectioned stained with haematoxylin and eosin method for determination of trabecular density and osteoclast-osteoblast proportion using ImageJ software (NIH -USA).

2.9 Statistical Analysis.
Statistical analysis was performed using Statistical Package for Social Science software (SPSS version 19.0; SPSS). Results were expressed as mean ± standard error of the mean (SEM) were analyzed using one-way ANOVA followed by Tukey's method. A statistical probability of $p < 0.05$ was considered significant.

3. Results
Qualitative phytochemical screening of methanolic extract showed the presence of saponins, flavonoids, tannins, steroids, and triterpenoids which may be responsible to ameliorate oxidative and inflammatory interventions in bone disorders.

3.1 Body weight and uterus weight
The mean body weight was 243±21 gr, after 4 weeks treatment, no noticeable increase was observed in their body weight. However, the weight of the uterus significantly dectreased in the OVX rats compared to those with intact ovaries ($p<0.05$). (Table 1).

Table 1. Comparison of body weight and uterus weight among different group

Groups	Body weight (g)	Uterus weight (g)
Sham operated (CMC 0.5% in 3 ml.kg b.w)	216.92±19.18	0.91± 0.51
Negative control (CMC 0.5% in 3 ml.kg b.w)	248.84±21.67	0.29±0.13
Positive control (ALD 0.18 mg/kg b.w	262.29±28.49	0.27±0.03
Low dose POE (300 mg/kg b.w)	243.83±25.33	0.32±0.07
Medium dose POE (600 mg/kg b.w)	238.46±19.58	0.26±0.04
High dose (900 mg/kg b.w)	248.59±15.01	0.31±0.22

*ALD : Alendronate b.w : body weight

3.2 Trabeculae density
To understand the extent of bone loss suffered by OVX rats, trabeculae density was evaluated indistal femurs and proximal tibia with Image J software. The density of trabeculae decreased averagedly 47% in the low dose (300 mg/kg b.w) group and medium dose (600 mg/kg b.w) group compared to sham group ($p<0.05$). However, treatment of the OVX rats with high dose (900 mg/kg b.w) POE group significantly incrreased the trabeculae density ($p<0.05$). Nonotheless, no statistically significant difference was observed among positive control, sham and POE high dose (900 mg/kg b.w) regarding the trabeculae density. The increase dose of POE extract was followed by an increase in trabecular density, so it can be concluded that the administration of the extract affects the density of trabeculae in ovariectomized rats (Table 2, Figure 1).

3.3 Osteoclast and osteoblast proportion

The proportions of osteoclast to osteoblast increased averagedly 87% in Negative control, lower dose (300 mg/kg b.w) and medium dose (600 mg/kg b.w) group. However, treatment the OVX rats with high dose (900 mg/kg b.w) POE significantly decreased the proportion of osteoclast to osteoblast. The increasin the dose of POE extract decreased the proportion of osteoclasts to osteoblasts in ovariectomized rats (Table 2, Figure 2).

Table 2. Comparison of trabeculae density and proportion of osteoclast to osteoblast among different group

Groups	Trabeculae density	Proportion of osteoclast to osteoblast
Sham operated (CMC 0.5% in 3 ml.kg bw)	71.43±16.79^{b}	7.24±3.11^{b}
Negative control (CMC 0.5% in 3 ml.kg bw)	38.79±16.79^{a}	12.93±3.83^{a}
Positive control (ALD 0.18 mg/kg b.w)	59.71±16.79^{b}	8.20±2.94^{b}
Low dose POE (300 mg/kg b.w)	36.46±16.79^{a}	12.94±5.03^{a}
Medium dose POE (600 mg/kg b.w)	40.05±16.79^{a}	14.25±8.05^{ac}
High dose (900 mg/kg b.w)	53.23±16.79^{b}	10.44±4.01^{c}

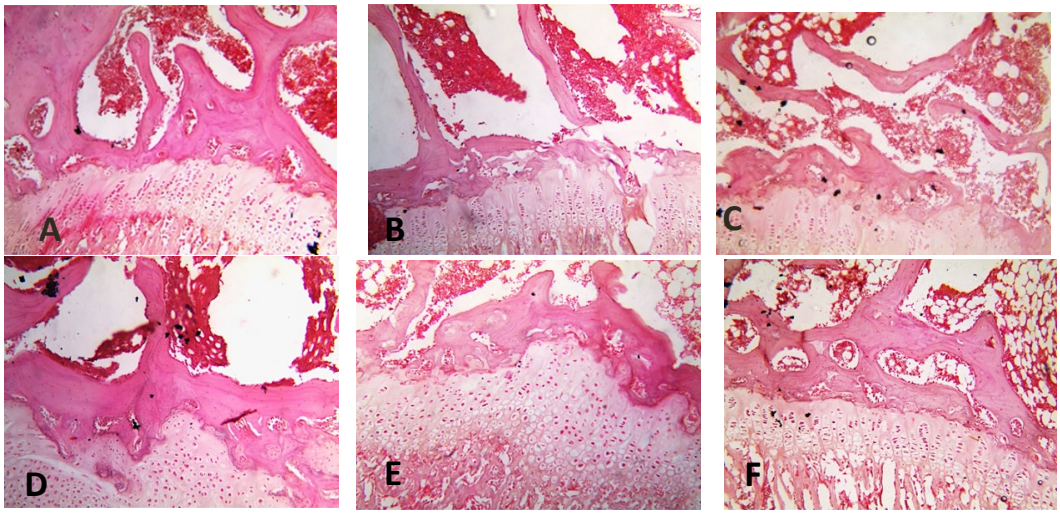

Figure 1. Histomorphometri of trabeculae density (Magnification 100X)

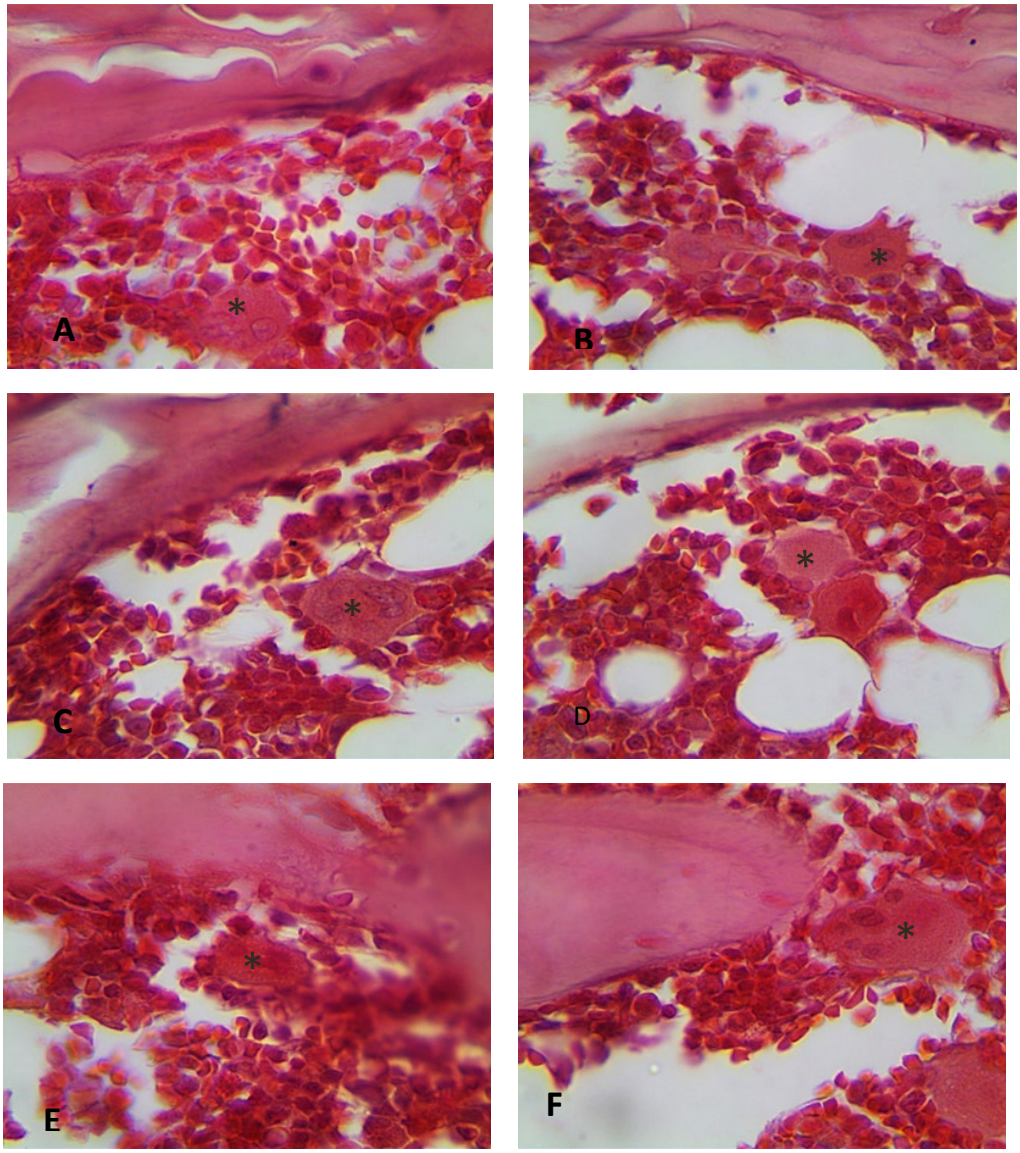

Figure 2. Microphotography of osteoclasts (asterisks) Magnification 1000X.

A. Sham group : B. Negative control (CMC – Na) : C. Positive control (Alendronate) ; D. Low dose (300 mg/kg b.w) ; E. Medium dose (600 mh/kg b.w); F. High dose (900 mg/kg b.w).

4. Discussion

Postmenopausal osteoporosis is the common form of osteoporosis associated with significant morbidity, mortality, deterioration in the quality of life and financial cost ((Kawai et al. 2011). Lower estrogen levels can increase the risk of bone loss and osteoporosis for females, so estrogen deficiency is a major cause of osteoporosis in postmenopausal women. Therefore, ovariectomized rats were regarded as an idea model of osteoporosis. In this study, the OVX rat model to asses the effect of POE on estrogen-deficiency induced osteoporosis. Current study demonstrated that OVX resulted in estrogen deficiency, uterine atrophy and bone loss, whereas exogenous POE supplementation rescue uterine atrophy and OVX bone loss. POE supplementation did not alter uterine weight, but rescue bone loss induced by OVX.

In the present sudy we evaluated the effect of four week POE supplementation on bone microarchitecture in ovariectomized rats based on bone histomorphometry. Histomorphometry is an important technique for examining bone quality and architecture (Lee et al. 2000). The four-week study period was chosen based on previous studies which showed that the changes in bone turnover markers were detected as early as the fourth week after exposure to oxidative stress (Ahmad et al. 2005).

The result of our study revealed that the trabecular density in ovariectomized negative control, lower dose (300 mg/kg b.w) and medium dose (600 mg/kg b.w) were significantly lower than in positive control, sham and high dose (900 mg/kg b.w). Ovariectomized rats also had higher separation compared to other rats (Figure 2). Studies have shown that the withdrawal of estrogen following ovariectomy induce perforation in trabecular plates and loss of connectivity density which results in the deterioration of trabecular bone micro-architecture (Aslam et al, 2010). In this study, treatment of the OVX rats with high dose (900 mg/kg b.w) POE group significantly increased the trabeculae density ($p<0.05$). However, no statistically significant difference was observed among positive control, sham and POE high dose (900 mg/kg b.w) regarding the trabeculae density. The increase dose of POE extract was followed by an increase in trabecular density, so it can be concluded that the administration of the extract affects the density of trabeculae in ovariectomized rats.

Bone mass is normal bone tissue is generally maintaned by a dynamic balanced between osteoclast bone resorption and osteoblast bone formation.The maintenance of bone mass depends on the dynamic balance between osteoblast osteogenesis and osteoclast bone resorption (Kim et al. 2018). The results of current study showed that 4 weeks treatment with POE supported the previous study in which it would undergo the cell changes of osteoblasts and osteoclasts in rat mandibular bone Sprague Dawley, in addition, it is influenced by the content of the Pandanus odoratissimus seed methanol extract, which is reported in previous studies that the flavonoids compounds have a positive effect on the bones with a stimulating effect on the activity of osteoblasts through estrogen- mediated action (Yao et al, 2008). The current study demonstrated that treatment of the OVX rats with high dose (900 mg/kg b.w) POE group decreased the proportion of osteoclasts to the number of osteoblasts.

Previous studies also showed that estrogen deficiency-induced oxidative stress with reduced antioxidase levels and activity in mouse bone tissue, and impaired osteogenesis and osteoblastic bone formation. The antioxidant NAC administration resulted in intense improvement in osteogenesis and osteoblastic bone formation in OVX mice (Shi et al. 2015) Current study confirmed that estrogen deficiency could induce oxidative stress and reduced antioxidase levels and activity, whereas POE supplement could reduce oxidative stress by increasing antioxidase levels and activity in OVX mice. Results from previous and this recent study, therefore, support that in vivo, estrogen plays a role to stimulate the osteoblastic bone formation and inhibit the osteoclastic bone resorption, at least partially through defense against oxidative stress.

Antioxidant rich plant, such as *Pandanus odoratissimus* may represent a possible approach for slowing down age-related bone mass reduction and enhancing bone remodeling. Rahayu et al (2020) showed that POE has higher antioxidant capcity than ascorbic acid. This correspondence to the higher amount of polyphenols in POE. Phenolic compounds are found to be associated with many health benefit (Parr and Bolwell 2000). They exhibit a wide range of physiological properties such as anti-inflammatory, antidiabetic, hepatoprotective, antimicrobial and antiviral (Adkar and Bhaskar 2014).

To the best of our knowledge, we are the fisrt to report the ability of POE in preventing osteopenia in postmenopausal osteoporosis rat model. The beneficial effect of POE on bone microarchitecture may be due to its antioxidant property. We propose that the antioxidant activity in POE prevented the free-radical-induced bone loss associated with estrogen deficiency. However, considering the result of this current study, future tresearch should focus on isolated or mixture of active constituents to determine the mechanisms underlying the bone effect and to reveal the beneficial therapeutic and safety properties of its phytochemicals, as a complementary and alternative medicine for management osteoporosis.

5. Conclusions

To sum up, oxidative stress may promote the development of osteoporosis, and antioxidants such as *Pandanus odoratissimus* seed extract can mitigate the progress of osteoporosis. Nevertheless, we made this conclusion based on the rat model of osteoporosis and *Pandanus odoratissimus* seed extract should be evaluated for adverse as well as beneficial effects in clinical trials. In addition, the specific mechanism of oxidative stress in osteoporosis should be further investigated.

Acknowledgment

We thank RistekBrin-Indonesia for PDUPT (Penelitian Dasar Unggulan Perguruan Tinggi) No. 163/E.41/Ak.04.PT/2021 grants.

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