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Effects of *Labisia pumila* on bone turnover markers and OPG/RANKL system in a rat model of post-menopausal osteoporosis

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SUMMARY

The loss of bone mass due to the increasing age in women is caused by the decline in ovarian function, known as osteoporosis. Numerous studies have evaluated the benefits of *Labisia pumila*, but not investigated the OPG/RANKL system. The purpose of the present study was to analyze the effects of *L. pumila* extract on bone turnover markers and OPG/RANKL system in ovariectomized rats. Totally, twenty-five female Wistar rats were divided into five tgroups (n = 5), including the control group, ovariectomized group, ovariectomized group treated with *L. pumila* extract of various doses (10 mg/kg; 20 mg/kg and 40 mg/kg). *L. pumila* extract was administered daily for 8 weeks. Serum levels of osteocalcin, urine levels of deoxypyridinoline, serum levels of OPG and RANKL were analyzed by means of ELISA technique. Serum levels of deoxypyridinoline and urine levels of osteocalcin were significantly higher in the ovariectomized group than those of the control group ($p < 0.05$). All doses of *L. pumila* significantly reduced serum levels

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of osteocalcin compared to the ovariectomized group, reaching the levels comparable to those of the control group ($p < 0.05$). Only the highest dose of *L. pumila* significantly increased the levels of deoxypyridinoline relative to the ovariectomized and control groups ($p < 0.05$). The levels of OPG and RANKL and the ratio of OPG/RANKL did not differ significantly among the treatment groups ($p > 0.05$). In conclusion, the ethanol extract of *L. pumila* normalized osteoblastic bone formation, but could not prevent collagen degradation in bone in a rat model of post-menopausal osteoporosis.

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1. Introduction

Reduction the bone mass and micro-architectural deterioration that triggering bone fragility and increased risk of fracture is characteristic of osteoporosis [1]. The loss of bone mass due to the increasing age in women is caused by the decline in ovarian function. Pathophysiology of the ovary-related bone loss is complex, and cannot be explained simply by increased bone resorption or decreased bone formation [2].

A bilateral ovariectomy model resembles accelerated bone loss in postmenopausal women, which is constituted by estrogen deficiency [3]. Bilaterally ovariectomized animals show an increase in osteoclastic bone resorption and reactive osteoblastic bone formation with the net result of a loss of bone mass [4]. When the receptor activator of nuclear factor-kappaB ligand (RANKL) expression is more dominant than that of osteoprotegerin (OPG), a progressive bone resorption ensues. When RANKL and OPG expression is comparable or OPG expression is higher than that of RANKL, bone neof ormation potentially takes place [5,6]. In detail, the protective effect of bone by OPG not through induction of bone formation to the normal value or improve the microarchitecture, but through inhibition of bone resorption and increase mineral composition [7–9].

Labisia pumila is a plant with very high phenolic and flavonoid levels. Both of these compounds in the plant are believed to have varied pharmacological activities. In communities of Southeast Asia, decoction of this plant is used as a health supplement to control irregular menstruation and menstrual pain, as well as a tonic to vagina walls after childbirth [10,11]. Traditionally, *L. pumila* extracts made by boiling the roots, leaves, or whole parts of the plant are useful to induce labor and metabolic function as an anti-obesity [12]. Numerous studies have evaluated the benefits of *L. pumila* in a rat model of osteoporosis [11,13–15], but no research has investigated the OPG/RANKL system. Hence, the purpose of the present study was to analyze the effects of *L. pumila* extract on bone turnover markers and OPG/RANKL system in ovariectomized rats.

2. Material and methods

2.1. Subjects

Totally, twenty-five female Wistar rats were acclimatized and randomized to five treatment groups: the control group (sham surgery), the ovariectomized group, ovariectomized group treated with *L. pumila* extract of various doses (10 mg/kg, 20 mg/kg and 40 mg/kg). The rats were maintained in room temperature (24 °C) and 12 h of light and 12 h of dark. In addition, the rats were fed standard laboratory diet and provided with *ad libitum* access to water.

2.2. Procedure for ovariectomy

After acclimatization, the rats performed anesthesia with ketamine (50 mg/kg) and xylazine (8 mg/kg), bilaterally ovariectomized group were subjected to ventral incision ovariectomy. In addition, five

control rats were subjected to sham surgery. Subsequent to ovariectomy, wound care was performed for 10 days and then extract treatment was started [15,16].

2.3. Preparation of extracts

Preparation of *L. pumila* extracts was performed according our previous study [15,17]. The ethanol extract of *L. pumila* was administered according to the dose using an oral feeding tube to the ovariectomized rats. *L. pumila* extracts were administered for 8 weeks. Determination of the dose was based on a previous study stating that the dose of *L. pumila* with no side effect was 50 mg/kg [18].

2.4. Serum and urine collection

At the end of the study, all rats were anesthetized and their blood collected from their hearts by cardiac puncture. Subsequently, the blood was centrifuged to obtain serum. In addition to serum, urine was also collected. Urine was collected by accommodated in metabolic cages. The urine is taken from the urine within 24 h.

2.5. Analysis of bone turnover markers

Serum bone formation markers were analyzed. For bone formation markers, analysis of the levels of osteocalcin used the Rat Osteocalcin/Bone Gla Protein of ELISA kit with the Catalog Number of E-EL-R0243 from Elabscience (Wuhan, Hubei, PRC). Analysis of urine bone resorption markers used the Rat DPD (Deoxypyridinoline) of ELISA kit with the Catalog Number of E-EL-R0327, purchased from Elabscience (Wuhan, Hubei, PRC).

2.6. Analysis of OPG and RANKL

Analysis of the levels of OPG and RANKL was performed on serum using ELISA kits purchased from Elabscience (Wuhan, Hubei, PRC). The analysis was performed according to the detailed procedures set forth in the kit.

2.7. Ethics

This study has passed the ethics review of the Ethics Committee, Faculty of Medicine, University of Brawijaya, Malang, East Java, Indonesia.

2.8. Statistical analysis

All data were presented in mean \pm SD. Differences in levels between treatment groups were analyzed by ANOVA and Least Significance Different using SPSS 16.0 statistical package. A p value of <0.05 was considered statistically significant.

3. Results

Figure 1 presents the serum levels of osteocalcin in each experimental group. Serum levels of osteocalcin were significantly higher in the group of rat model of post-menopausal osteoporosis than those of the control group ($p < 0.05$). All administered doses of *L. pumila* lowered serum levels of osteocalcin significantly compared to the group of rat model of post-menopausal osteoporosis, reaching levels comparable to those of the control group ($p < 0.05$).

In this study, urine levels of deoxypyridinoline were significantly higher in the group of rat model of post-menopausal osteoporosis than those of the control group ($p < 0.05$). The levels of

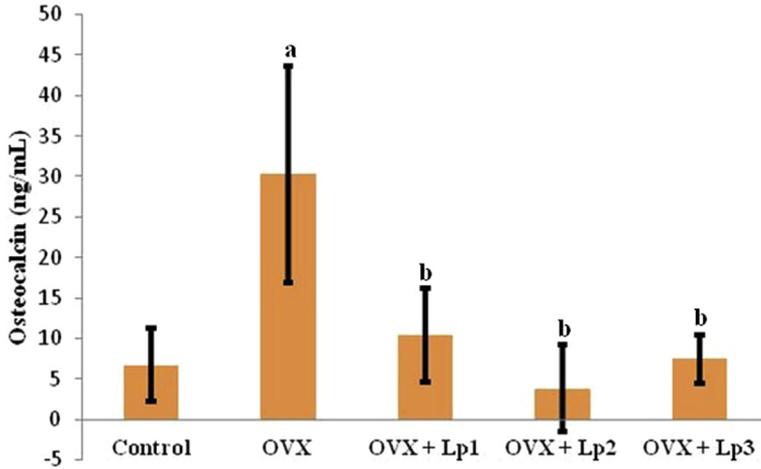


Fig. 1. Osteocalcin level in serum of control and experimental groups. Note: values are presented as mean \pm SD; ^a $p < 0.05$; in comparison with control (untreated) group; ^b $p < 0.05$; in comparison with ovariectomized rats (OVX) group; OVX + Lp: ovariectomized rats treated by *Labisia pumila*; ng/mL: nanogram/milliliter.

deoxypyridinoline were significantly higher in all groups of *L. pumila* treatment than in the control group. Nonetheless, only the highest dose of *L. pumila* increased the levels of deoxypyridinoline significantly compared to the group of rat model of post-menopausal osteoporosis and the control group ($p < 0.05$), as seen in Fig. 2.

Table 1 presents the levels of serum OPG, RANKL, RANK: OPG ratio in each experimental group. The levels of OPG and RANKL did not differ significantly among treatment groups ($p > 0.05$). Besides, the RANK: OPG ratio also did not differ significantly among treatment groups ($p > 0.05$).

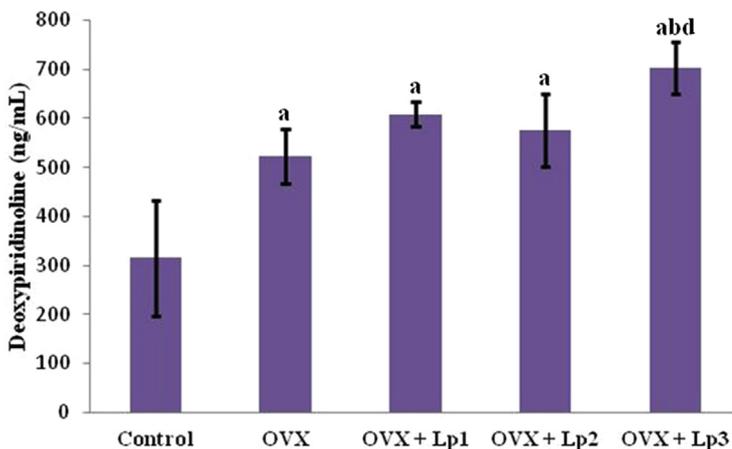


Fig. 2. Deoxypyridinoline level in urine of control and experimental groups. Note: values are presented as mean \pm SD; ^a $p < 0.05$; in comparison with control (untreated) group; ^b $p < 0.05$; in comparison with ovariectomized rats (OVX) group; ^d $p < 0.05$; in comparison with second dose of *Labisia pumila* group; OVX + Lp: ovariectomized rats treated by *Labisia pumila*; ng/mL: nanogram/milliliter.

Table 1

Levels of serum OPG, RANKL and RANKL: OPG ratio in each experimental group.

Level	Control	OVX	OVX + <i>Labisia pumila</i>		
			10 mg/kg BW	20 mg/kg BW	40 mg/kg BW
OPG (pg/mL)	904.35 + 310.38	272.95 + 284.73	437.68 + 263.09	577.32 + 816.80	237.90 + 215.59
RANKL (pg/mL)	23.62 + 14.51	97.35 + 57.60	75.53 + 20.29	75.14 + 45.56	76.18 + 57.63
RANKL/OPG	0.032 + 0.027	0.796 + 0.631	0.271 + 0.259	0.403 + 0.460	0.760 + 1.064

Note: Values are presented as mean \pm SD; OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor-kappaB ligand; pg/mL: picogram/milliliter; OVX: ovariectomized rats; mg/kg BW: milligram/kilogram body weight.

4. Discussion

In the present study, serum levels of osteocalcin were significantly higher in the ovariectomized group than those of the control group ($p < 0.05$). This indicated that approximately nine weeks after ovariectomy in rats, osteoblasts remained acting to form bone as a compensatory mechanism to inhibit the rate of bone loss due to estrogen deficiency. This finding was consistent with those of the previous studies that bilaterally ovariectomized animals showed reactive osteoblastic bone formation characterized by a net result of a loss of bone mass [4]. The increase in the levels of osteocalcin was caused by increased formation of osteoblasts, rather than as a result of increased production by osteoblasts [19]. This finding was in contrast to those of the previous studies showing decreased levels of osteocalcin [14,20]. This contrast was caused by the ratio of osteocalcin secreted into the blood and bound to the hydroxyapatite crystals [20]. Interestingly, there was no significant difference in the levels of OPG among treatment groups ($p > 0.05$). This finding indicated that the reactive osteoblasts as a characteristic of osteoporosis due to ovariectomy did not involve an increase in the production of OPG which would inhibit the activation of osteoclasts. Previous studies found no change, increase, or decrease in OPG in the similar experiment [21–24]. In this study, levels of RANKL and the ratio of RANKL: OPG tends to increase in ovariectomy group compared to the control group, although it has not yet reached significant differences. This indicates that the existing mechanism of bone loss, played by RANKL as a trigger differentiation and activation of osteoclasts.

Meanwhile, urine levels of deoxypyridinoline were significantly higher in the ovariectomized group than those of the control group ($p < 0.05$). Deoxypyridinoline constitutes the cross-linking collagen molecules as a result of post-translational modifications of collagen. Deoxypyridinoline is more specific in bone and not affected by diet or internal metabolism [25]. This marker is a bone resorption marker [26]. This indicated that in ovariectomy bone collagen was degraded to a higher extent than that of the control. This was also in accordance with the characteristics of osteoporosis due to estrogen deficiency, such as an increase in osteoclastic bone resorption [4]. Osteoclastic resorption in this model was not activated by elevated levels of RANKL, but other biofactors [27].

All doses of *L. pumila* reduced the serum levels of osteocalcin significantly compared to those of the ovariectomized group, reaching the levels comparable to those of the control group ($p < 0.05$). This demonstrated that the active components of *L. pumila* acted through normalizing the reactive-compensating osteoblasts in bone formation to achieve the formation comparable to that of the control group. We also hypothesized that this decrease was caused by the bound to the hydroxyapatite crystals [20]. The findings of the present study were inconsistent with those of the previous studies that found an increase in the levels of osteocalcin due to administration of *L. pumila* [14]. This contrast was caused by differences in dosage and type of extracts. However, the highest dose of *L. pumila* increased the levels of deoxypyridinoline significantly compared to those of the ovariectomized group and controls ($p < 0.05$). This indicated that the higher dose of *L. pumila* administered to rat model of post-menopausal osteoporosis, may restore osteoblast function to normal, but led to increased bone collagen degradation. We hypothesized that in OVX induced osteoporosis, *L. pumila* could not prevent collagen degradation in bone. This finding is controversial with that of the previous studies, but with a different marker of bone resorption, the C-terminal telopeptide of type 1 collagen [14]. In this study, the extract *L. pumila* tend to increase levels of OPG, suppress levels of RANKL, and the ratio of RANKL: OPG, although it has not yet reached significant differences. This indicates that the existing

mechanisms of inhibition of bone loss through normalization of OPG and RANKL by active ingredient *L. pumila*.

In conclusion, the ethanol extract of *L. pumila* normalized osteoblastic bone formation, but could not prevent collagen degradation in bone in a rat model of post-menopausal osteoporosis.

Competing interests

We declare that we have no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.yclnex.2018.01.002>.

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