

***Citrus reticulata's* Peels Modulate Blood Cholesterol Profile and Increase Bone Density of Ovariectomized Rats**

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Abstract

Hormon Replacement Therapy is a common therapy for estrogen deficiency but in other side it will increase the risk of cardiovascular disease. Another alternative therapy which relatively more safe is using phytoestrogen. The *Citrus reticulata's* peel contain flavanone and polimethoxyflavone which are suspected to give estrogenic effect, therefore it is potential to be used as phytoestrogen. The purpose of this study was to examine the estrogenic effect of *Citrus reticulata's* peel extract in modulation of bone density and blood cholesterol profile of ovariectomized rats (OVX), an animal model of postmenopausal osteoporosis.

Thirty six 7-weeks-old female Sprague Dawley rats were assigned to six groups: a SO group, an OVX group, an OVX+CMCNa group, an OVX+extract dose 500 mg/kgBW group, an OVX+extract dose 1000 mg/kgBW group, and an OVX+estradiol group. After 7 weeks, the rats were killed then blood and femoral were collected immediately. The rontgenogram indicated that extract and estradiol administration increase the bone density. And the data analysis with Oneway ANOVA test, followed by Shceffé test ($P < 0.05$) showed that extract can improve blood cholesterol profile in dose depend manner. These results suggest a possible role of *Citrus reticulata's* peel extract as women's health agent because of its beneficial effects on bone and lipids.

Keywords : *Citrus reticulata*, estrogenic , bone density, blood cholesterol profile.

Introduction

Estrogen modulates all women's body physiological function, from modulate menstruation cycle and reproduction until modulate bone density and cholesterol transport (Jorand, 2004). Estrogen deficiency in a menopause woman will increase osteoclast cells in bone tissue causing osteoporosis (Meiyanto *et al.*, 2001), and also increase LDL level that can be a risk to cause coronary disease. Moreover, estrogen deficiency will decrease the quality of women life because it can trigger health disruption and cause unpleasant symptoms, like *hot flashes*

and insomnia (Jorand, 2004). Therefore, the women need estrogen addition from outside to keep the estrogen balance and function in the body.

The alternative effort to overcome estrogen deficiency is using Hormone Replacement Therapy (HRT). This therapy effectively decreases the unpleasant menopause symptoms. The hormone will bind to estrogen receptor (ER) then modulate bone density and blood cholesterol profile. However, HRT can induce breast cancer development, stroke and also blood clots (Jorand, 2004). Therefore, we need the substitute of estrogen which relatively safe. One of the alternatives is using phytoestrogen, estrogen-like compounds

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from plants, such as flavonoid (Benassayag *et al.*, 2002). Estrogen modulates all women's body physiological function, from modulate menstruation cycle and reproduction until modulate bone density and cholesterol transport (Jorand, 2004). Estrogen deficiency in a menopause woman will increase osteoclast cells in bone tissue causing osteoporosis. Estrogen modulates all women's body physiological function, from modulate menstruation cycle and reproduction until modulate bone density and cholesterol transport (Jorand, 2004). Estrogen deficiency in a menopause woman will increase osteoclast cells in bone tissue causing osteoporosis (Meiyanto *et al.*, 2001), and also increase LDL level that can be a risk to cause coronary disease. Moreover, estrogen deficiency will decrease the quality of women life because it can trigger health disruption and cause unpleasant symptoms, like *hot flashes* and also increase LDL level that can be a risk to cause coronary disease. Moreover, estrogen deficiency will decrease the quality of women life because it can trigger health disruption and cause unpleasant symptoms, like *hot flashes* *Citrus reticulata's* peels have unique flavonoid compounds, there are flavonol, flavanon, and polimethoxyflavone such as tangeretin, hesperidin, hesperetin, rutin, nobiletin, naringin and naringenin (Choi *et al.*, 2007). These compounds mostly possess estrogenic effect, that can be used as phytoestrogen.

Adelina *et al.*, (2008) reported the ethanolic extract of *Citrus reticulata's* peels performed estrogenic effect in ovariectomized rats. The extract triggered the proliferation of breast epithelial cells and increased uterus weight which may due to the interaction between ER and the *Citrus's* peels flavonoids. This interaction will increase ER activation, which showed in blocking of osteoclast differentiation, bone resorption (Murkies *et al.*, 1998), and also

improving the LDL metabolism (Gent *et al.*, 2004) through increasing the LDL receptor (Stavreus *et al.*, 2001). In this study, we examined the estrogenic effect of extract ethanolic of *Citrus reticulata's* peels in the modulation of blood cholesterol profiles and remodulation of bone density in ovariectomized rats.

Materials and Methods

Animals

The 7-weeks-old female Sprague Dawley rats were obtained from UPHP (Unit Pengembangan Hewan Percobaan) UGM. After a 3-days adaptation period, 36 rats were either sham-operated (n=6) or oOVX (n=30). The rats were housed in group plastic cages and were given the same food and distilled water.

Preparation the *Citrus reticulata's* peels extract

The healthy, green-yellowish, and half-ripened *Citrus reticulata* were obtained from Tawangmangu, Central Java. The fruits were determined in Pharmacognocny laboratory, Faculty of Pharmacy UGM with Flora of Java book to check the species reference. The fruits were washed and the peels were removed, dried and extracted with ethanol 70% for 5 days with maseration methode. The supernatant were filtered and dried up by rotary evaporator until the viscous and brown extract was obtained.

Estrogenic assay

The 36 rats were divided into 6 groups randomly, namely SO, OVX, OVX+CMCNa, OVX+extract dose 500 mg/kg, OVX+extract dose 1000 mg/kg, and OVX+estradiol (EST) groups. The sham-operated (SO) group and one group of OVX rats were not given any treatment except food and distilled-water. One group of OVX rats received CMC-Na (OVX+CMC group) as the control group. Two groups of OVX rats

receive the extract which one group received 500mg/kgBW extract dose (OVX+EJK500) and the other were received 1000mg/kgBW extract dose (OVX+EJK1000). The last group of OVX received 17- β -estradiol (OVX+EST). All groups were given treatments for 7 weeks whether in second-week the blood were collected and the end of experiments all the animals were sacrificed.

Blood Cholesterol Analysis

Serum were incubated in room temperature for 15 minutes then centrifugated 4000 rpm for 20 minutes to collect the serum for the blood cholesterol total, triglyceride, and HDL analysis. The blood cholesterol total concentration were determined by enzymatic-colorimetric method where 10 μ l of serum were added by 1 ml cholesterol reagent then shaken slowly and left it in room temperature for 20 minutes then the absorbance were measured at λ 546 nm. The triglyceride concentration were determined by enzymatic-colorimetric method where 10 μ l of serum were added by 1 ml triglyceride reagent then shaken slowly and left it in room temperature for 20 minutes then the absorbance were measured at λ 546 nm. The HDL concentration were determined by lipoproteins precipitation, except LDL, VLDL, and chylomicron then followed by enzymatic-colorimetric method. The precipitation reagent is 0,2 ml of Mg^{2+} + 0,5 ml HDL precipitant which contain $MgCl_2$ (25 mmol/L) and phosphotungstic acid (0,55 mmol/L). The mixed of the solutions were shaken slowly then left in room temperature for 10 minutes then centrifugated 1200 rpm for 2 minutes. The precipitant were separated then the HDL concentration were measured by spectrophotometer which 0,1 ml of supernatant were added 1 ml cholesterol reagent then shaken slowly and left in room temperature for 20 minutes then the absorbance were measured at λ 546 nm. For

the measure of LDL concentration, we choose this formula :

$$\text{LDL cholesterol (mg/dl)} = \text{blood cholesterol total concentration} - \text{presipitant concentration}$$

Bone density analysis

The femoral bone were carefully removed at necropsy. The right femur of each rat was used for analysis with rontgen in animal hospital UGM. The rontgenogram of each groups was analysis and compared with other treatments. The evaluation were done qualitatively and were examined by 5 people.

Statistic analysis

The total cholesterol, triglyceride, HDL, and LDL data were analyzed with SPSS 15.0, ANOVA method then followed by Shceffé test to know the difference significance.

Results and Discussion

Blood cholesterol profile

Estrogen modulated the blood HDL, LDL, and triglyceride. Intake of extract for 2 weeks did not affect the blood total cholesterol can be observed with comparing the blood cholesterol profile the two of extract dose groups with OVX+CMC group as the negative control and OVX+EST as the positive control (Figure 1). Extract could significantly decrease the LDL concentration with dose dependent manner. In other words, higher extract dose the lower LDL concentration, but still higher than estradiol effect. Intake of the extract could increase the HDL profile but not significantly, as well as OVX+EST group. This finding showed neither the estradiol nor the extract affect the HDL profile of ovariectomized mice significantly ($P < 0,05$) (Figure 1.).

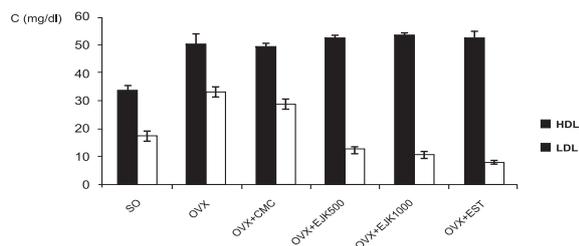


Figure 1. LDL and HDL concentration after treatment in SO, OVX, OVX+CMC, OVX+EJK500, OVX+EJK1000, dan OVX+EST groups. The value showed mean \pm SD, n=4, a showed the significant value in Scheffè test ($P < 0,05$) compared with SO group and b showed the significant value in Scheffè test ($P < 0,05$) compared with OVX+CMC group.

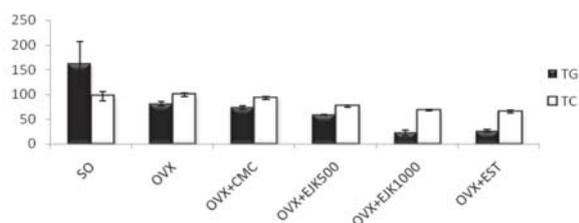


Figure 2. Triglyceride and Total Blood Cholesterol after treatment in SO, OVX, OVX+CMC, OVX+EJK500, OVX+EJK1000, dan OVX+EST groups. The value showed mean \pm SD, n=4, a showed the significant value in Scheffè test ($P < 0,05$) compared with SO group and b showed the significant value in Scheffè test ($P < 0,05$) compared with OVX+CMC group.

Bone density analysis

The OVX+Extract1000 group rontgenogram showed that extract could increase the epifisis's femur density compared with OVX+CMC group and OVX group (Figure 2C). The potency of the extract was lower than estradiol (Figure 2C-2D).

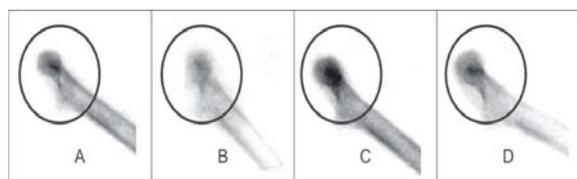


Figure 3. The bone density measured by rontgenogram profile in treatment groups; ovariectomy (OVX), CMC-Na treatment (OVX+CMC), Extract 1000mg/kgBW dose (OVX+Extract1000), and estradiol treatment (OVX+EST) of ovariectomized rats. (A) OVX, (B) OVX+CMC, (C) OVX+EJK1000, and (D) OVX+EST.

In this experiment, we want to show the extract potency to modulate the blood cholesterol profile, like decrease the HDL concentration and increase the LDL, triglyceride, and total cholesterol. Besides that, the extract also increased the bone density of ovariectomized rats. The two of these estrogenic effect complete the *Citrus reticulata's* peels extract's estrogenic effect. In previous experiment, the extract could improve the breast's epithelial cell proliferation and also increase the uterus volume through c-Myc expression (Adelina *et al.*, 2008 and Supriyati *et al.*, 2008). This experiment also strengthen the proven that extract could be a phytoestrogen.

The using ovariectomized rats is a model for the estrogenic research which the rats condition are in estrogen deficiency and did not have any supply except the addition estrogen from outside body. The ability of the extract to modulate the blood cholesterol profile and increase the bone density of ovariectomized rats in this experiment indicated the *Citrus reticulata's* peels extract could be used as the substitute of estrogen/hormone replacement therapy (HRT). The extract's estrogenic effect are expected through some mechanisms which was beginning from the phytoestrogen-ER binding in cytoplasm or nucleus. The complex of phytoestrogen-ER would activate ER then dimerization occurred and activate the *estrogen response elements* (ERE) (Meyer *et al.*, 2006).

In decreasing LDL concentration, the complex of phytoestrogen-ER will bind with ERE in LDL receptor, Sp-1 or Ap-1 that was the promoter of LDL receptor (Brüning *et al.*, 2003 and Lundeen *et al.*, 1997). The complex of the three components will improve the expression of LDL receptor then increase the LDL metabolism causing the blood LDL concentration decreased (Joles *et al.*, 1997). The increasing of blood HDL was initiated by the complex of phytoestrogen-ER to activate the enhancer

protein apo A1 (Harnish *et al.*, 1998). The complex of these components will bind with the promoter apo A1 then trigger the *apo A1* gene expression, the main substance of HDL, will increase (Joles *et al.*, 1997). Bok *et al.* (1999) proved that naringin and hesperidin, the citrus's peels flavonoid, could decrease the total cholesterol concentration and blood triglyceride with blocked the activity of HMG-CoA reductase of rats leading to inhibition of the cholesterol synthesis in the body (Chiba *et al.*, 2003).

The re-modulation of bone density by the *Citrus reticulata's* peels extract were explained by the inhibitory mechanism of osteoclast differentiation and activation. The activated osteoclasts were mediated by the binding of the activator NF- κ B (RANK) with its ligand (RANKL). RANKL bind to RANK in osteoclast membrane and stimulated the bone resorption which can cause osteoporosis. In bone remodeling, the phytoestrogen will act like estrogen in increasing the osteoprotegerin (OPG) expression in osteoblast. The OPG is the decoy receptor of RANKL and compete actively with RANK in binding RANKL. More OPG expression will decrease the osteoclast activation and this phenomena will prevent osteoporosis (Min *et al.*, 2000).

Overall, the *Citrus reticulata's* peels extract had a potency to be developed as the menopause women healthcare agent. Beside that, the extract need to be utilized for the health because *Citrus reticulata* was a local fruit and easy to be cultivated. From this experiment, we need more experiments to know the optimum dose of the extract as a phytoestrogen. The using and application of the extract suggest very good for estrogen deficiency, for the example the menopause women.

As the conclusion of this research, the *Citrus reticulata's* peels extract increase the bone density and improve the blood cholesterol profile of the female ovariectomized rats.

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