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The Combination of High Calcium Milk with *Citrus maxima* Peels Ethanolic Extract Increased Bone Density of Ovariectomized Rats

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Abstract

Osteoporosis is a common problem in menopause woman. The main cause is lack of estrogen hormone. Commonly, prevention therapy is by consuming high calcium milk, but it is not effective. Bali orange's peel (*Citrus maxima* Merr.) is a waste material but known contains phytoestrogen according to previous study. Considering of this result, fortification of high calcium milk and Bali orange's peel is expected to be an effective solution for osteoporosis in menopause woman. This research began with extraction of Bali orange's peel (BPE) using ethanol 70% by maceration method. Ovariectomized Sprague Dawley female rats as the model of post menopausal woman were treated by BPE for 28 days. The doses of BPE was given to rats is 500 and 1000 mg/KgBW combined with high calcium milk. Bone density was determined using digital microradiography, the profile showed the increase of bone density in group that treated with combination of BPE 1000 mg/Kg BW and high calcium milk compare to control and given only milk groups. Docking molecular showed that BPE's active compound which are hesperidin and naringin have interaction with estrogen receptor α and β. Docking score of naringin with ER α and β are -19,97; -18,99 respectively. Meanwhile the docking score of hesperidin with ER α and β are -19,98; +49,92 respectively. Overall, the result of this research showed that fortification of BPE with high calcium milk has good prospect to develop as effective therapy of osteoporosis.

Keywords : *Citrus maxima* Merr., phytoestrogen, osteoporosis, high calcium milk, estrogen receptor

INTRODUCTION

Osteoporosis is a disease that characterized by reduction in bone tissue mass per unit volume that effect thinner and fragile bone and less number of calcium contain (Liliana, 2000). The report from WHO says that in Asia, fracture caused by osteoporosis will increase from 84.000 cases in 1984 to 6.26 million cases in 2050. Almost 71% of all cases happen in the developing countries (Sankraan, 2000). One of two women in the world has a risk to have osteoporosis syndrome. The high incidence of osteoporosis in women cause this condition becomes a major health problem in society.

Women at menopause state have higher risk to osteoporosis. Menopause is natural process that will be experienced by every woman. In this state, the function of ovaries to produce estrogen hormone will decrease. Resulting in a variety of physiological change include osteoporosis (Kenny *et al.*, 2000). Decrease of estrogen production will be followed by increase calcium lost in woman body (Perry and O'Hanlan, 2003). Estrogen regulates OPG (Osteoprotegerin) that have a role in differentiation of macrophage to osteoclast which is affect in calcium metabolism pathway.

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Regulation of osteoclastogenesis by OPG is to inhibit interaction between RANKL (Receptor Activated NF-κB Ligand) and its receptor that resulting decrease number of osteoclast formation (Sculi*et al.*, 2000).

Nowadays, osteoporosis treatment for post-menopausal woman is consumption of high calcium milk and calcium supplement. By this treatment, the deficiency of calcium is decrease but osteoporosis still can occur (Noordin, 2009). Data from nutrition survey says that 30 – 40% of post-menopausal women in Indonesia consume high calcium milk, but still can be found high prevalence of osteoporosis case in Indonesia (Arifin, 2010). This shows that therapy has not been effective by milk consumption.

Osteoporosis is not just caused by lack of calcium intake but also due to a decrease in estrogen production. One of therapy to prevent estrogen deficiency is the substitution of natural compounds that relatively safe in the form of phytoestrogens. Phytoestrogens are nonsteroidal chemical compounds in plants that have estrogenic activity (Yildiz, 2005). Bali orange's peel (*Citrus maxima*) or BPE contain flavonoid naringin and hesperidin which has been proven as phytoestrogen (Choi *et al.*,2007).

The estrogenic effects of BPE are increase bone density, modulate cholesterol blood profile, increased breast cell proliferation and increased uterine weight (CCRC unpublished data, 2011). High calcium milk was commonly used as osteoporosis therapy. Therefore, we did fortification using high calcium milk and BPE. Fortification is the addition of nutrients obtained or intentionally added from outside and not from the original food (BPOM,2003).

As the initial study was to know estrogenic effect of BPE, we did molecular docking to identify the interaction between estrogen receptor with phytoestrogen that contained in BPE. The results from this method become the basic for *in vivo* study to determine the effects of combination of high calcium milk and BPE in ovariectomized rat *Sprague Dawley* strains. The results of this research can be the basis for the formulation of high-calcium milk with BPE and also solve the problem of osteoporosis in postmenopausal women.

MATERIALS AND METHODS

Plant and extract preparation

Citrus maximas were collected from Jedigan, Trirenggo, Bantul. The sample was determined at the laboratory of plant taxonomy, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia. Citrus maxima peels were collected, dried using oven under temperature 40^{o} C, then made into powder using grinder, and extracted with 70% ethanol using maceration method. Macerate was concentrated using a rotary evaporator to obtain viscous extract (BPE).

Animals Preparation

Thirty eight female Sprague-Dawley rats, age 40-50 days were obtained from UPHP (Unit Pengembangan Hewan Percobaan) Universitas Gadjah Mada, Yogyakarta, Indonesia. The animals were grouped and housed in cages at temperature and humidity-controlled room (25-32^{o} C and 98% relative humidity) and given free access to food and water. The animals were given time to adapt with laboratory condition for 10 days.

Bone density analysis

The femoral bones were carefully removed at necropsy. The right femur of each rat was used for qualitative and quantitative analysis. Qualitative and quantitative analysis were done by measuring bone density using microradiography digital in Imaging Laboratory, Faculty of Science, Universitas Gadjah Mada.

Molecular Docking

The program that we used for molecular docking was PLANTS 1.1. Estradiol was used as native ligand to compare the ligand bond strength. Test compounds or test ligands were naringin, hesperidin. Then molecular docking was performed between 17β-estradiol (native ligand), naringin, hesperidin to Estrogen Receptor α and Estrogen Receptor β. Scoring was done for the docking results.

Data Analysis

The compound or test ligand that has lowest score in molecular docking showed the strongest affinity to receptor. Bone density data were analyzed by Excel MS Office 2007 and SPSS 17 One way ANOVA was used to assess concentration ($p<0.05$) then post-hoc comparisons were made using Tukey significant different test.

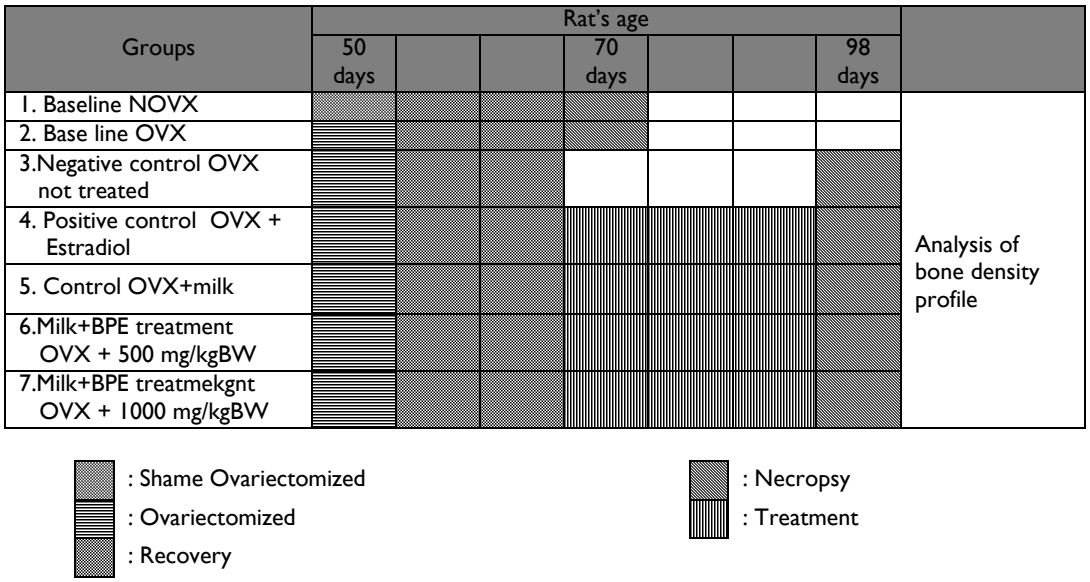


Figure 1. Experimental design. The rats divided into seven groups of 5-6. The groups were baseline non ovariectomy (baseline OVX), baseline ovariectomy (baseline OVX), solven control (OVX+CMC-Na), positive control (OVX+Estradiol), control milk (OVX+High calcium milk), Milk+BPE treatment dose 500 mg/kg BW (OVX+BPE500) and treatment with Milk+BPE dose 1000 mg/kgBW (OVX+BPE1000). Baseline groups were sacrificed at 70 days old, while other groups were start given treatment. Treatments were given for 28 days. At the end of experimental period, animals were sacrificed, and then femoral bones were collected.

RESULT AND DISCUSSION

Identification of BPE Flavonoid Content Using Thin Layer Chromatography

According to Choi *et al.* (2007) Bali orange's peel (BPE) contain flavonoid such as naringenin, hesperidin, rutin, tangeretin, and nobiletin. To identify flavonoid content of BPE, we used thin layer chromatography (TLC). From TLC assay qualitative type of flavonoid contained in BPE can be seen. Reference compound that we use is rutin, naringenin, and hesperidin.

Some information that we can see through TLC profile are polarity and type of compound in BPE. Polarity of compound can be determined using Retention factor (Rf) whereas the identification of compound through colour change after spray reaction using $AlCl_3$ under UV 366 nm.

The main characteristic of flavonoid compound, according to Wagner *et al.* (2000) is fluorescence in green-yellow color under UV 366 nm. So that, it can be seen that BPE contain flavonoid compound. Based on TLC profile obtained can be conclude that the flavonoid compounds that have been identified in BPE are rutin, naringenin, and hesperidin. It showed by the similarity of hRf value from each spotand the color of fluorescence after observed under UV 366 nm.

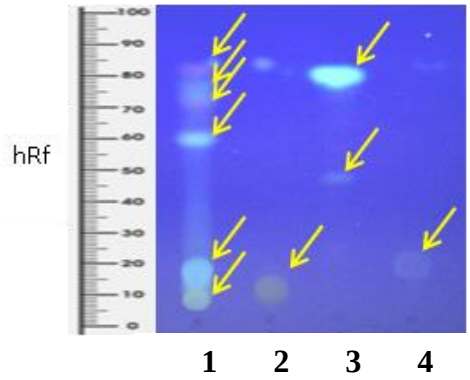


Figure 2. TLC profile of BPE after elution. Using silica gel GF 254 as stationary phase and mobile phase ethyl acetate : methanol : formic acid (95 : 5 : 0,5% v/v/v). (1) BPE ; (2) Rutin ; (3) Naringenin ; (4) Hesperidin. There are some spots of BPE that have the same hRf with reference compound but in different colour intensities. From TLC profile known that hesperidin is one of major compound of BPE. After spray reaction and observed under UV 366 nm, some spots which fluorescence in green-yellow color indicating flavonoid compound.

Effectiveness of the addition of BPE on High Calcium Milk to Increase Bone Density Ovariectomized Female Rats

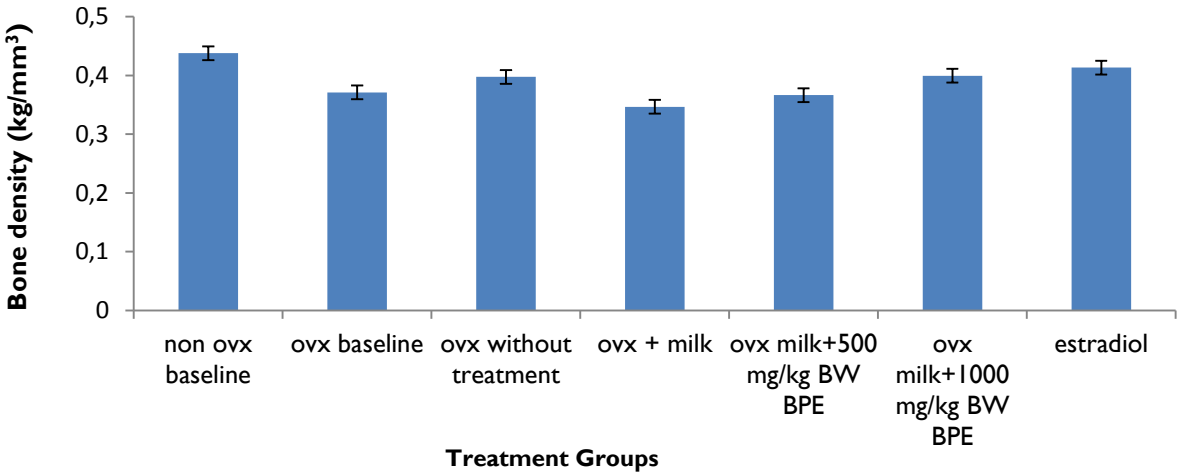


Figure 3. Bone density profile from each group after 28 days treatment. Data show quantitative bone density. Value indicates mean from each group + standard error bar. Bone density profile from each treatment groups did not differ significantly (P>0.05) by ANOVA followed by Tuckey test. However, there is a tendency to increase the bone density of treatment of milk and BPE at dose 1000 mg/kg BW when compared with the untreated control and control milk group.

Female rats were ovariectomized to obtain the model of post-menopausal woman condition. After ovariectomy, test animal will experience estrogen deficiency and not receive estrogen intake apart from BPE or estradiol for the positive control. This treatment was done in order to determine the estrogenic effect of BPE in increasing bone density (Fig. 3)

Bone density at baseline OVX group decreased compared to baseline non-OVX indicating that ovariectomy surgery has successfully carried out in this study. Based on statistic test, it is found that the bone density profile of the treatment groups was not significantly different from each group.

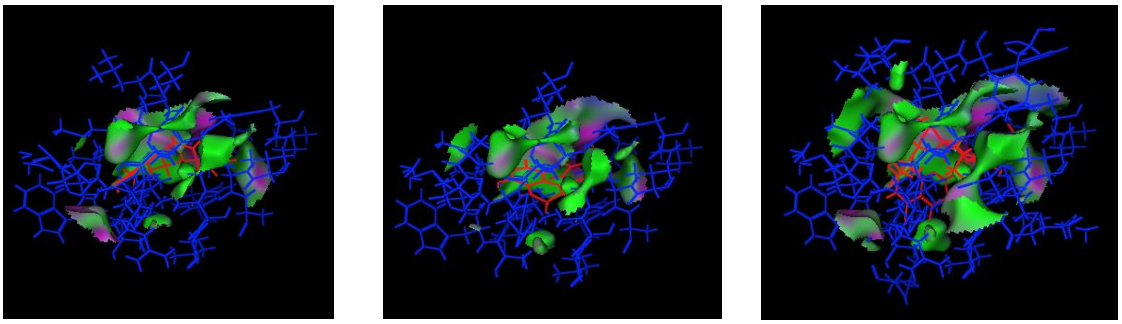
However, from graphic above we can see the trend of increasing bone density of the group treated with a combination of milk and BPE at higher doses compared with the control milk. This indicates that the BPE potentially improve the effectiveness of milk in increasing bone density.

The results showed that administration of a combination of high calcium milk with EJB in general has the potency to increase bone density on ovariectomized female rats. In the state of estrogen deficiency, calcium in the body can not improve bone density optimally. This is due to the imbalance of osteoblast and osteoclast activity. Deficiency of estrogen makes the activity of osteoclast increase. By the addition of BPE as estrogenic substances source can optimize the maintanance of bone density. This mechanism is mediated by inhibition of osteoclast activity through osteoprotegerin (OPG), which is a decoy receptor in the activation of osteoclasts.

Interaction of Estrogen Receptor α and β to Flavonoid Content of BPE by Molecular Docking

Tabel 1.*Docking score*in ERα dan ERβ

Ligan	*Score*	
	ERα	ERβ
Estrogen (17β-estradiol)	-20,00	-20,00
Naringin	-19,97	-18,99
Hesperidin	-19,98	+49,12



(A) **(B)** **(C)**

Figure 4. Docking visualization using MOE application. Describe interaction between ligand and ERβ. Ligand (A) 17β-estradiol; (B) naringenin; (C) hesperidin showed in red image interact with receptor in the same active site. Reported that the bonds between the ligand and the receptor are hydrogen bond symbolized by the pink and green color through the hydrophobic bond.

Docking is done to predict the ability of the active compounds (ligands) to interact with estrogen receptors (ERα and ERβ). Estrogen is an intracellular receptor that is one of the regulators on calcium metabolism. If activated by ligands, estrogen receptor will affects osteoclast differentiation via formation of osteoprotegerin (OPG).

Native ligand used is 17β-estradiol to compare the ability of the bond or affinity of naringin and hesperidin with estrogen receptor. The parameters used are docking score that represents the energy required to bind the receptor. The lower scores obtained by docking the stronger bonding compounds with ligands. Score docking compounds on estrogen receptor ligand can be seen in Table 1.

Docking results showed that naringin and hesperidin have a similar affinity to 17β-estradiol against Erα. This is indicating with similar of docking score to ERα. However, the docking results showed that only naringin Erβ having docking score similar to 17β-estradiol, while docking score hesperidin has a much higher. Thus, only naringin which has

affinity with Erβ, showed by docking score that not significantly different with native ligand. Docking score differences on hesperidin showed selectivity in estrogen receptor activation. This makes hesperidin to develop as potential selective estrogenic substance. Strong activation of estrogen receptor β will trigger uterine cancer in women. The docking results corroborate previous research that says that the flavonoid compound is estrogenic.

Based on this study, the combination of BPE and high calcium milk could potentially increase bone density ovariectomized female rats because of the phytoestrogens are able to bind to Estrogen Response Element (ERE) as evidenced by molecular docking.

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