The Effect of Ethanolic Extract of Purple Yam Tuber (Dioscorea alata L.) on Bone Calcium Levels in Ovariectomized Rat

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Abstract
This study is on the effects of the ethanolic extract of purple yam tuber (Dioscoreaalata L.) on the bone calcium levels in ovariectomized (ovx) rat models of osteoporosis. Forty-five of 8-weeks-old female Sprague–Dawley rats were randomly assigned to six groups as followed: sham-operated, OVX, OVX-Estradiol, OVX-EDA 116 mg/kg BW, OVX-EDA 232 mg/kg BW, OVX-EDA 463 mg/kg BW for 30 days. The administration of EDA and estradiol was given orally using a stomach tube.It is on the 31st day since the bones of femur and Tibia rat were isolated. The isolated bones were analyzed using wet destruction and the bone calcium level was measured using the AAS. The bone calcium level were tested statistically using one way ANOVA.The average of bone calcium levels for each group: for the normal group is 18,30 $\pm$ 3,17%, OVX 16,44 $\pm$ 3,93%, OVX-Estradiol 16,59 $\pm$ 2,63%, and for the group of OVX-EDA dose 116; 232; 463 are 15,89 $\pm$ 1,66%, 15,96 $\pm$ 2,61%, and 14,91 $\pm$ 2,31% respectively. The conclusion is that the treatment of purple yam tuber (*Dioscoreaalata*L.) with the different dose of 116; 232; and 463 mg/kg BW for 30 days cannot increase the bone calcium level in the ovariectomized rats. According to the statistical data there is no significant differences among the treatment groups.
Keywords: Calcium, .*Dioscorea alata*, Extract

INTRODUCTION

Osteoporosis is a condition where the bones are weak and it is breakable. Osteoporosis is one of the bone metabolic diseasesignaled by the decreasing of the bone mass because of the deficit of the bone matrix and mineral along with the damage of the microarchitecture of the bone tissue that causes the decreasing of the bone strength and the risk of bone fracture. [1]*National Osteoporosis Foundation* states that 44 millions American have the risk to get osteoporosis, and 22% of them are adults up to senior people who mostly are menopause women. [2]

Menopause is a condition when the ovariums are unable to produce estrogen. Estrogen hormone has contribution to help the the calcium absorption in the intestine to prevent the bone density. The decreasing of the calcium absorbtion will disrupt the balance of the calcium in the blood because the low blood calcium may lead to the increasing of the calcium reabsorption in the bone that causes low bone mass. The decreasing of the calcium level in the bone because of the estrogen deficiency is called *postmenopausal osteoporosis* or *osteoporosis tipe*I.[3]

The injection of the hormone to substitute the estrogen (*Estrogen Replecement Therapy*-ERT) is commonly given to the menopause women to overcome the symptom that occur in pasca-menopause and to prevent chronic disease like osteoporosis.[4] The sintetic estrogen preparat given as the therapy to substitute the hormome is expensive as well as it has bad side effects. Syntetic estrogen can interfere the blood coagulation process, disturb enzyme works in the liver, lead to endometrium uterus bleeding, nausea and throw up. [5]

The use of syntetic estrogen preparate continuously can cause carcinoma ovarium. [6] Besides, the use of estrogen and progesterone preparate as the hormone replacement therapy can also lead to the breast cancer. [7]

There are side effects in the hormonal therapy. It is wiser to use phytoestrogen therapy as the alternative for the menopause women because it is saver.[8] Phytoestrogen is a natural substance that can influence the body

estrogenic activities. [9]

One of the plants that has phytoestrogen substance is the purple yam tuber (*Dioscorea alata* L.). The D tuber has 5 substances that has the estrogenic quality based on the *ligand-dependent*[10] transcription activities. The consumption of *D. alata tuber* can increase the strength of bones in the ovariectomized rats. [11]

Testing the effect of ethanolic extract of *D. alata*on the calcium level of ovariectomized rats was conducted in this research and measured by using AAS. This reseach is hoped to be able to prove the effect of ethanolic extract of *D. alata* tuber on the increasing bone calcium level in the ovariectomized rats. Therefore, the tuber is useful to be used as the phytoestrogen agent to prevent the osteoporosis in the menopause women. The result of the research is also useful as the reference in the utilization as well as for other researcher in the pharmachology division. This research aims to know the effect of ethanolic effect of *D. alata* tuber on the bone calcium level in the ovariectomized rats.

MATERIALS AND METHODS

Plant and Chemical Materials

Purple yam tuber (*Dioscoreaalata* L.)*D. alata*used in the present study were collected from the commercial market in Kulonprogo Yogyakarta and authenticated at the Laboratory of Pharmacognosy, The Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia. Concentrated nitric acid solution, Perchlorate acid solution, distilled water and Calcium Carbonate were obtained from LPPT Gadjah Mada University, Yogyakarta, Indonesia

The Preparation of Ethanolic Extract of Purple Yam Tuber (*Dioscoreaalata* L.)

The dried powders of tuber *Dioscoreaalata* L were extracted by maseration using ethanolic 70 %. The ethanolic extracts were evaporated to obtain the concentrated extract.

Animals

Female Sprague–Dawley rats, aged 6 week, were purchased from Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. The animals were grouped and housed in polyacrylic cages with not more than five animals per cage and maintained under the standard laboratory conditions (temperature 25 ± 2°C) with dark and light cycle (12/12 h) and allowed free access to commercial pellet diet and water ad libitum.

Administration Procedure

Rats were acclimatized to laboratory condition for 1 week before the commencement of the experiment. Ovariectomized were done to all experimental animals except to the normal group. Animals were assigned to the experimental groups, normal (sham-operated), OVX, OVX-Estradiol, OVX-EDA116 mg/kg BW, OVX-EDA 232 mg/kg BW, OVX-EDA 463 mg/kg BW, with five animals per group, per experiment. Twenty days after ovariectomy, all of the rats were allowed to the controlled access to a commercial standard pellet and free access to deionized water for 20 days.

The extract of *D. alata* tuber was given orally once a day during 30 days using the sonde technique and the doses are 116 mg/kg BW, 232 mg/kg BW, and 463 mg/kg BW to the experiemental group of OVX-EDA. The group of OVX-Estradiol was given estradiol with the dose of 2 µg/day, and the control groups and OVX did not get the treatment. On the 31st day, the rats were sacrificed to take their thigh bones.

The Measurement of Bone Calcium Level Using the Atomic Absorption Spectroscopy (AAS)

The isolated bones then were weighted to find out the weight of the bones. The sample of the isolated bones that had been weighted was to be destructed by using 15 ml HNO_3 and 3 ml $HClO_4$, and heated until the bones dissolve in the solution and the solution became clear. The destruction was continued until $\pm$3 ml left, then added 25 ml of distilled water to it. The solution of the sample was filtered and diluted using the distilled water until 100 ml. The sample as the result of destruction process was ready to be read at the absorbance part using the AAS at the wafelength of (λ) 422,7 nm. The result of the reading then was compared with the standard curve, therefore came up the level of bone calcium in mg/dl or ppm.

The standard curve is the result of the absorbansi measurement from several series dilution from calcium standard dilution. The concentrations of carbonat calcium ($CaCO_3$) that was used as the standar solution are 0; 0,05; 0,1; 0,2; 0,5; 0,8; 1; 2; 3; 4; and 5 mg/L. The result of standar solution absorbance measurement would create the similarity on the linear lines that were used to find out the calcium level of the ovariectomized rat bones that were used as the sanple,

Data Analisis

The normality of the bone calcium level data were tested using the *Saphiro-Wilk*. The results of the normality and varian test data wereanalized using *One Way* ANOVA test, and the measurement was continued using the Tukey test to know the meaningful differences among the treatment groups.

RESULT AND FINDING

The average of bone calcium level was gained from the measurement of bone calcium level inwhite rats (*Rattus norvegicus*) type *Sprague-Dowley*ovariectomy model. Those rats were given the teraphyof etanolic extract of purple yam tube *Dioscorea alata* L) and measured by AAS. The result is shown in Table 1.

Tabel 1. The average of bone calcium level.

No.	Group Treatment	The Average of Bone Ca (%) Level
1.	Normal	18,30 ± 3,17
2.	OVX	16,45 ± 3,94
3.	OVX-Estradiol	16,59 ± 2,63
4.	OVX-EDA dosis 116 mg/kgBW/day	15,89 ± 1,66
5.	OVX-EDA dosis 232 mg/kgBW/day	15,96 ± 2,61
6.	OVX-EDA dosis 463 mg/kgBW/day	14,91 ± 2,31

Ovariectomy is a process to take away the ovarium in order to make the model rats become menopause. The assumption is that after the ovariectomy, the rats will experience the decreasing of estrogen hormone and it is similar to the hormone deficiency in menopause women. The decreasing of the estrogen hormone after the ovariectomy because the ovarium as the hormone producer is alredy been removed. The decreasing of estrogen hormone in the animal model is proven by Hartiningsih*et et al.,* (2010)[12] who explains that the ovariectomy in rats can decrease drastically the estrogen hormone level, so the condition is similar to menopouse.

The bone calcium level is different between normal group and OVX group. The normal rat treatment group is the groups that have normal ovarium and they still produce estrogen endogen hormone. The average of bone calcium level at the normal group is 18.30%, and it is higher than OVX group, 16.45%. Although the difference is not significant based on the statistic analysis. The decreasing of bone calcium in the OVX group is to prove that that estrogen deficiency in the ovariectomy rats also decrease the bone calcium level. Nurrochmad *et al.,* (2010)[2] also state the same result.

Estrogen endogen deficiency in the menopause women can lead to the increasing of osteoclastogenesisproces and the loss of bone mass. In order to keep the estrogen balance, menopause women need to get substitute for the estrogen hormone. Estrogen intake can increase the apoptosis of osteoclasts as it hinders the formation of cytokins that can stimulate the osteoclasts cell differentiation (IL-1, IL-6, dan TNF-α). Besides, the estrogen intake can also stimulates the *osteoprotegerin* (OPG) ekspression and TGF-β (*Transforming Growth Factor*-β) from the osteoblast and stroma cells, so the process can hamper bone resorption.[13,14] It is in line with the result of calcium level in OVX-Estradiol, which get the Estradiolteraphy of 2 µg/day. The average of the calcium level is 16.59% and it is higher than the OVX group.

The mechanism of bone resorption which is mediated by osteoclast cell will damage the bone mineral crystals ligament that havethe form of *calcium phosphatase* with the collagen matrix. The interaction of osteoclast with the bone surface during the process of resorption not only release some acids (for example citrit acid, lactat acid) from the mitochondria and secretory vesicles, but also release the hydrogen ions through *carbonat anhidrase* dan *vacuolar ATPase pompa proton* channel. This condition creates acidic environment at the *resorptive pit* that cause the damage on the bone matrix.[15] Osteoblast cells have a role to secret the bone matrix component like collagen and proteoglycan. Collagen has function to give strength to matrix and binding the bone mineral salt. Proteoglycan has function to deposite the bone mineral. [15]

Calcium and phosphate salts are deposited on the surface of the bone maker cells as the non-crystal compound which then are converted into *hydroxyapatite* [$Ca_{10}(PO_4)_6(OH)_2$] of the bone major crystal salt. The function of *hydroxyapatite* is to keep the ion and give strength to the bone. Some *hydroxyapatites* stay in the form of non-crystal to fascilitate the fast absorption when needed. [15]

The averages of bone calcium level from the treatment rat group of OVX-EDA 116; 232; 463 are 15.89%; 15.96%, 14.91% repectively. The averages are smaller than OVX and OVX-Estradiol groups. The intakes of *D. alata* tuber extract for 30 days do not give effect to the significant increasing of the calcium level.

D. alata tuber contains fitoestrogen that is useful for the menopause women. Fitoestrogen in *D. alata* tuber is different from the existing fitoestrogen, namely diosgenin. Diosgenin in *D. alata*tuber is spirostanolsaponin that is composed from hydrolific sugar and bounded at the hydrophobic steroid aglycone. The effect of estrogenic from diosgenin is used as the major precursor of the synteticstereoid production. [16] It has been proven before that the effect of the work mechanism of the estrogenic diosgenin is almost the same as the estrogen hormone, which is to influence the formation process and bone resorption seen from the result of RANKL/OPG by Zhang *et al.,* (2014).[17]

The inconsiderable impact posed by the increasing bone calcium level can be caused by many factors that can influence the bone formation process. One of the factors is the inadequate dose of the ethanolic extract of *D. alata* purple yum tuber. Zhang *et al.* states that the intake of diosgenin dose 96 mg/kg BW for 12 weeks

can give the effect of antiosteporosis in menopause women. Another research says that the content of diosgenin in 100 g *D. alata* is only 82.39 mg diosenin. [18] Therefore the intake of ethanolic extract of *D. alata* purple yum tuber with the doses of 116; 232; 436 mg/kg Bw a day does not give effect of antiosteoporosis in increasing the bone calcium level because the doses are inadequate and the time is also short. Bone calcium level is not only influenced by estrogen hormone, but it is also influenced by other factors, such as calcitonin, parathyroid hormone and the intake of vitamin D that have role to manage the osteoclasts and osteoblasts function. The result of ANOVA statitistic test shows that the bone calcium levels among the group are not significantly different; it is shown form the significant value of p>0,05 (0,554). Furthermore, the *Tukey* test also shows that significant values of all the samples above are more than 0.05, which means that all of the data on the bone calcium levels are not different actually among the treatment.

CONCLUSION

This research prove that the intake of ethanolic extract of purple yum tuber (*Dioscorea alata* L.) with variety of doses 116; 232; and 463 mg/kg BW during 30days cannot increase the bone calcium level in the ovariectomized rats. Another research should be conducted on the effect of ethanolic extract of purple yum tuber (*Dioscorea alata* L.) on the ovariectomizedrats with higher doses and longer treatment.

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