

## Efficacy of Water Clover (*Marsilea crenata*) Extract Against Blood Estrogen-Progesterone Balance, Blood Calcium Levels and Impact on Dense of Bone Tissue of Rat (*Rattus norvegicus*)

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### ABSTRACT

The potential of phytoestrogen for the stabilized of low condition blood estrogen hormone much more important to be studied. The aim of this research was to find a natural herbal active compounds Water Clover Extract (*Marsilea crenata*) which contain phytoestrogen isoflavones as an alternative invasive treatment against menopause women and sub estrus in the dam of livestock. This research is true experimentally post-test control design based on completely randomized design. The old females white rat (*Rattus norvegicus*), divided into five groups challenges with 2 ml each *Marsilea crenata* extract in a different concentration such as 6.25%, 12.5%, 25%, 50% given by gastric sonde along 23 days, compare negative control group. The study showed that *Marsilea crenata* extract plays a role in increasing toward blood calcium levels and increase bone density in the animal laboratory (*Rattus norvegicus*), supported by blood estrogen-progesterone balance. The *Marsilea crenata* extract could be potential as a substance to increase estrogenic progesterone balance and raise blood calcium both in human and animal.

**Keyword:** phytoestrogen; Blood Calcium; dense of bone tissue.

### INTRODUCTION

Phytohormone has already used in many cases as an alternative treatment against infectious and non-infectious

disease attack both human and animals. Already widely researched about many bioactive substances just like coumestan, and lignan, but that there were much wider such as daidzein and genistein that have different functions (Kim and Park, 2012). The estrogenic potency of isoflavones is about 100-fold (coumestrol) to 1,000-fold (daidzein) weaker than that of  $17\beta$ -estradiol. Phytoestrogens are a substrate of the plants that have estrogen-like activity (Glover et al., 2006), have properties are compounds produced by plants that have properties similar to estrogen, although different chemical structures. This substance is naturally occurring estrogen found in Group plants with components similar to the hormone estrogen (Setchell, 1998). The chemical compound including a group of phytoestrogens are isoflavones, coumestans, and lignans. The group is daidzein and isoflavones genistein (Kim and Park, 2012). The chemical compound including a group of phytoestrogens are isoflavones, coumestans, and lignans. The group is daidzein and isoflavones genistein (Kim and Park, 2012).

Water clover (*M.crenata*) is one kind of water plants that grow in the rice fields. There are many water clovers grow in the most areas of Indonesia. Water clover has a mineral content in the leaves and stalks namely, potassium, phosphorus, iron, sodium, calcium, zinc, and copper. Water clover also contains phytochemicals such as alkaloids, steroids, flavonoids,

carbohydrates, reducing sugars, and amino acids. The content of minerals and phytochemicals that function as calcium oxalate crystals dissolve (CaOx) is potassium and flavonoids (Nurjanah, 2012).

Progesterone/Estradiol (P4/E2) ratio has been suggested as a marker for predicting clinical pregnancy rates, but the evidence is conflicting (Lai et al., 2009). Depending on ambient estrogen may act as estrogen agonists in a low estrogen environment (Morito et al., 2002). Therefore, isoflavones are also referred to as selective ER modulators (SERMs) (Chang et al., 2009).

The estrogen hormone is a steroid hormone, produced by the internal theca cells of ovarian follicles, corpus luteum, placenta and little is generated by the adrenal cortex (Ganong, 1995). Lack of estrogen will lead for increasing levels of the hormone parathyroid, thereby increasing bone resorption, resulting in decreased bone mass (Gruber et al., 2002). Calcium plays a role in the process of bone formation, muscle contraction, blood clotting and others. When a cow loses calcium as a result of the milking process, then the blood calcium must be replaced.

#### MATERIAL AND METHODS

The research was conducted in the Laboratory of Epidemiology, Faculty of Animal Husbandry, University of Brawijaya, Malang. Female *Ratus norvegicus* two months old and weight 100-150 grams, divided into five groups, namely the negative control group and four treatment groups. The four groups were treated by 2 ml each of *Marsilea crenata* extract in a different concentration such as 6.25%, 12.5%, 25%, 50% given by gavage along 23 days.

A sampling of blood serum was performed at the beginning and the end of the study research. The blood samples were centrifuged at a speed of 1,500 rpm for 6 minutes and put in a test tube then using Atomic Absorption Spectrophotometer for testing blood calcium levels (Karaman, 2013). Analysis of the blood calcium level uses atomic absorption spectrophotometric. Readings are then compared with the standard curve to obtain the level of calcium in mg/dl or ppm (Suarsana, 2011). The titer of Progesterone and estrogen used Elisa technique. Dense of bone tissue illustrated on Histopathological (HE staining) of os humerus were observed using a binocular light microscope to compare among treatment.

#### Statistical (CRD) analyzed

ANOVA Data analysis and MDRS. Results of histopathological preparations os humerus as a descriptive analysis.

#### RESULTS AND DISCUSSION

##### The Effect of Water Clover Leaf Extract (*Marsilea crenata*) on Blood Calcium

Phytoestrogen compound improves calcium balance especially in female rat. Their involvement with bone health is essential to the relationship between titer of blood estrogen and blood calcium. Blood Estrogen would supports intestinal absorption of calcium. The results of measurements of blood calcium levels in rats after administration of water clover leaf extract (*Marsilea crenata*) provoked to stimulate the amount of blood Ca concentrations compared to control (Table 1). This data correspond several studies, which isoflavone increased the level of calcium and estrogen in the blood (Suarsana, 2011; Zobda, 2013). This change occurred due to the provision of the extract containing compounds phytoestrogens such

as daidzein and genistein that has a structure resembling a 17-beta estradiol can occupy the estrogen receptor so as to cause effects like endogenous estrogen (Abid, 2005).

**Table 1. Result of treatment *Marsilea crenata* Extract in Blood Calcium level (P <0.05)**

Treatment	Blood Ca level mg/dl
K-	12.825 <sup>ab</sup>
P1	13.115 <sup>bc</sup>
P2	13.74 <sup>bc</sup>
P3	14.29 <sup>c</sup>
P4	15.075 <sup>d</sup>

#### Effect of Extract (*Marsilea crenata*) on Steroid Hormone

The data suggested that the administration of the extract was able to stimulate the level of progesterone and estrogen hormone in the blood (Table 2). The result slightly different compared to the previous report that the application 50%-200% of the extract for 60 days showed no significantly different (Suarsana, 2011). This is due to phytoestrogens have a very low affinity to the estrogen receptor than the endogenous estrogen, so it takes a high dose so as phytoestrogens able to cause estrogenic effects of endogenous estrogen withdrawal (Pawiroharsono, 1998). The upregulation of the estrogen and progesterone may have an impact on bone regulation (Apley, 1993). Estrogen regulates the activity of osteoclasts, which results in a slowing of dissolving old bone. On the other hand, progesterone promotes the production of osteoblasts that are required to form new bone. Progesterone has been shown to stimulate the new bone formation required to prevent and reverse osteoporosis (Morito et al., 2002).

**Table 2. Titer of Blood Estrogen and Progesterone After Treatment**

Treatment	Progesteron pg/ml	Estrogen pg/ml
K-	17.87 <sup>a</sup>	261.66 <sup>a</sup>
P1	16.66 <sup>a</sup>	333.00 <sup>b</sup>
P2	21.08 <sup>b</sup>	360.60 <sup>c</sup>
P3	21.40 <sup>b</sup>	361.33 <sup>c</sup>
P4	29.99 <sup>c</sup>	394.00 <sup>d</sup>

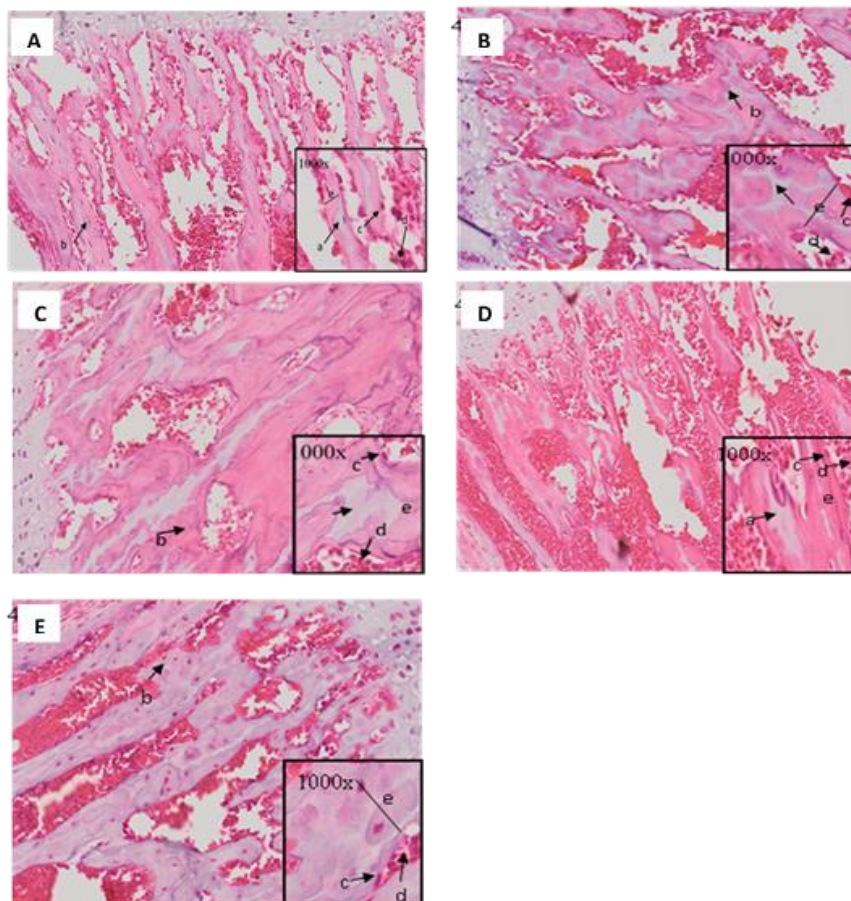
#### Dense of bone tissue

The histology data showed that the control group (Figure 1A) had no cartilage characterized by a white color, the osteocytes look flattened shape and bone matrix size smaller. The other hand, administration the extract repair the bone profile which is shown by the widening of the bone matrix as compared to the negative control group. In the 6.25% extract treated group (1B) demonstrated that bone matrix visible widening compared to negative control and cartilage was a presence in the matrix.

The administration 12.5% of the extract (Figure 1C) showed wider matrix dilation than control; and the persistence of the cartilage in the bone matrix, and the number of scattered osteocytes. The presence of osteoblasts which indicates the process of ossification or osteogenesis still occurs. Moreover, the 25% extract treated group (Figure 1D) showed bone matrix dilation; and the number of osteocytes that less than compared to the 12.5% extract treated group. The 50% extract treated group (Figure 1E) showed osteogenesis activity was higher compared with the groups, which are more visible dense bone matrix and reduced cartilage in the bone matrix, and showed many osteocytes. Bone remodeling occurs when the bones are damaged or have become old bone, which may ultimately

reduce the strength of the bone. Bone damage will be reabsorbed by the osteoclasts, osteoblasts cells after being absorbed and will form new bone to replace the bone that is dissolved by osteoclasts

(Cosman, 2009). At the end of the remodeling cycle, osteoblasts remain on the surface of the new bone, or into the matrix as osteocytes.



**Figure 1.** Histology humerus in control (A), treated with the extract 6.25% (B), 12.5 % (C), 25%(D), and 50% (E). Hematoxylin-eosin stained the tissue, then visualized by microscope in the magnification 400x. a (cartilage), b (osteocytes), c (osteoblasts), d (osteoclasts). The matrix (e) of treated mice is wider than control. The dilatation process on matrix occurs in all of the treated mice compared to the control, and the cartilage distribution also changes accordance with the dose of the extracts.

During the process of bone formation, osteoblast cells will be influenced by the availability of calcium and estrogen. At current levels of calcium, increases will stimulate the thyroid gland to produce hormones that suppress parathyroid hormone calcitonin to inhibit the formation of osteoclasts, so there will be an increase in

bone osteoblasts. Osteoblasts will then be transformed into osteocytes, bone cells today are deeply embedded in the one matrix (Junqueira and Carneiro, 2005).

The results showed that administration of the extract blood calcium levels, which influenced bone formation. Phytoestrogens can serve to replace the activities of

endogenous estrogen in increasing Ca absorption in the gut and increasing the Ca reabsorption in the kidney (Van Abel et al., 2003). The optimum condition was 50% concentration of *Marsilea crenata* extract that illustrated more osteoblast and osteocyte, which warrant for increasing bone density.

Estrogens are multi-functional hormones, and one of their functions involves the bones. The phytohormones influence bone metabolism through different mediators like growth factors. One such a growth factor is Insulin-Like Growth Factor-1 (IGF-1). The IGF-1 is a potent growth factor for osteoblasts. It also increases bone resorption and induces osteoblast apoptosis. Estrogen deficiency affects the increased levels of parathyroid hormone (PTH) and will increase bone resorption, resulting in a decrease in bone mass and accelerate the process of bone fragility.

### CONCLUSION

Administration of *Marsilea crenata* extract increased the titer of blood Ca and raise the rate of osteogenesis. Moreover, the study suggested that the extract could be used for low estrogen hormone therapy in the female. The clover leaf Extract (*Marsilea crenata*) plays a role in blood calcium levels and provide changes to bone density rat (*Rattus norvegicus*) becomes thicker at a concentration of 50%.

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