

## Activity of 70% Ethanol Extract of *Chrysophyllum cainito* in Increasing Vertebrae Trabecular Bone Density in Female Mice

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### ABSTRACT

Phytoestrogens are a class of compounds that have estrogen-like functions or structure. This research was conducted to find out the phytoestrogens activity in *Chrysophyllum cainito* leaves, in increasing the vertebrae trabecular bone density of female mice, using dexamethasone induction as osteoporosis model. The 70% ethanol extract of *C. cainito* leaves was given to female mice with dose of 100; 200; and 400 mg/kgBW mice/day after being induced orally with dexamethasone with 0.145 mg/kgBW mice/day. The positive control used was 1.3 mg/kgBW mice/day alendronate. This research had been done in four weeks. The increasing of trabecular vertebrae bone density of female mice was measured with Histomorphometry staining methods. The result showed the 70% ethanol extract of *C. cainito* leaves have activity in increasing trabecular vertebrae bone density for female mice with optimum dose of 400 mg/kgBW mice/day. This activity occurs because phytoestrogens content in 70% ethanol extract of *C. cainito* leaves, that may replace the function of estrogen in its bond with ER.

**Key words:** Phytoestrogen; *Chrysophyllum cainito*; Flavonoid; Osteoporosis

### INTRODUCTION

Osteoporosis is a condition of bone mass degradation along with microarchitecture damage which causes improvement of bone fracture risk (Ahmed and Elmantaser, 2009; Agrawal and Gupta, 2013). The microarchitecture damage in bone tissue is related tightly to the existence of turnover abnormality in the bone remodeling process, that creates unbalanced of bone formation and resorption process (Kini and Nandeesh, 2012; Lee *et al.*, 2013; Meeta, 2013). Estrogen deficiency is known as one of important factor that creates bone remodeling unbalance process for postmenopausal woman (Lee *et al.*, 2013; Dipiro *et al.*, 2008), and hormone replacement therapy (HRT) is a medical treatment which mostly recommended for osteoporosis in postmenopausal woman (Rachman *et al.*, 2004). Unfortunately, giving HRT in long-term therapy can create a potential side effect such as coronary event, venous thromboembolism, stroke, breast cancer, and dementia (Constantine and Pickar, 2005; Wibowo, 2009; Lee *et al.*, 2013).

Phytoestrogens are a class of compounds from the plant that can substitute the estrogen

function in its bound with estrogen receptor. Instead of easily found in plants and does not have the side effect, the phytoestrogens group were also reported to have benefits to increase the bone mass (Urasopon *et al.*, 2008; Villiers, 2009; Yang *et al.*, 2012). Then, it becomes the potential alternative medical treatment for postmenopausal woman (Yang *et al.*, 2012). *Chrysophyllum cainito* was known as plant which contains phytoestrogens. *C. cainito* grow a lot in East Java, Indonesia. People use its fruit to be consumed as food. However, this plant has not been widely studied. One of the studies showed the 70% ethanol extract of *C. cainito* leaves contained polyphenol, flavonoid, isoflavone, tannin, sterol, and triterpenoid compound group (Luo *et al.*, 2002; Koffi *et al.*, 2009). Isoflavone, sterol, and triterpenoid are compound which has an estrogenic activity that is included in the phytoestrogens compound.

To know the phytoestrogens activity of *C. cainito* leaves in fixing bone remodeling process, the mice (*Mus musculus*) trabecular vertebrae bone density level measurement was done by giving the treatment of 70% ethanol extract of *C. cainito* leaves, then the measurement of mice bone density level was done by histomorphometry and HE methods.

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Figure 1. Normal mice. (a) and mice with osteoporosis; (b) The change of vertebrae posture becomes kyphotic is shown by arrows.

## METHODOLOGY

### Materials

#### Plant Material

*C. cainito* was taken and identified in UPT Materia Medica, Batu, Indonesia in July 2017 with specimen number 1b-2b-3b-4b-6b-7b-9b-10b-11b-12b-13b-14a-15a-109b-119b-120a-121b-124b-125a-126b-127a. The fresh leaves were dried and grinded to produce green leaves powder.

#### Chemical

The 70% ethanol (Merck), dexamethasone (Kimia Farma), alendronate (Novel), CMC Na 0.5% and chloroform (Merck) were purchased from Phytochemistry Laboratory in Pharmacy Department, Faculty of Medical and Health Science, Maulana Malik Ibrahim State Islamic University, Malang. The 10% formalin, 10% formic acid, 70% alcohol, 3% nitrate acid, acetone, 1.5% xylol, liquid paraffin, glycerin, ammonia solution, hematoxylin and eosin dye were acquired from Histology Laboratory, Faculty of Medicine, Universitas Airlangga, Surabaya.

### Methods

#### Sample Preparation

*C. cainito* leaves were extracted with 70% ethanol with ultrasonic assisted extraction methods, and were prepared to produce extract suspension in aquadest with dose 100; 200; and 400 mg/kgBW mice/day. Dosage one was made by mixing 280 mg extracts to 50 ml CMC Na 0.5%. Dosage two was made by mixing 560 mg extract to 50 ml CMC Na 0.5%. Dosage three was made by mixing 1120 mg extract to 50 ml CMC Na 0.5%.

#### Treatment

Female mice were induced with dexamethasone of 0.145 mg/kgBW mice/day orally. Then each group was treated with 100; 200;

and 400 mg/kgBW mice/day samples, and 1.3 mg/kgBW mice/day alendronate as positif control. It had been given in four weeks orally for five groups (contains six mice in each group). The next step was mice surgery to take the trabecular vertebrae bone. The vertebrae bone was cut in thorax part and put inside the bottle with 10% formalin. Trabecular vertebrae bone then inserted in decalcification solution (7.0g aluminum chloride, 8.5 g chloride acid, and 5.0 ml formic acid in 100 ml water) and then the density were tested. Neutralization was done using sodium sulfate 2% for 24 hours. Trabecular vertebrae bone then washed using water for 12 hours and rinsed with 70% alcohol. The bone then blocked with paraffin and cut with microtome. Next step was hydration with 70% alcohol and hematoxylin as the main dye. The bone was inserted in 1% acid alcohol and ammonia solution, and was added with 1% eosin. The final step was clearing the bone with 1.5% xylol, and plated on object glass. The observation slide was analyzed using an Olympus Cellsens program with 100x zoom.

#### Data Analysis

The research result was analyzed using one-way ANOVA with a significance value of 0.05 ( $p \leq 0.05$ ). If there was any difference then would be tested by using post hoc testing with LSD to test the difference and average of the treatment.

## RESULTS AND DISCUSSION

Extraction of *C. cainito* leaves with 70% ethanol produces extracts with yield value of 13.92%. Osteoporosis was created with dexamethasone induction of 0.145 mg/kgBW mice/day orally for female mice. Mice with osteoporosis could be distinguished from healthy mice by looking at their crooked back (kyphotic) (Figure 1).

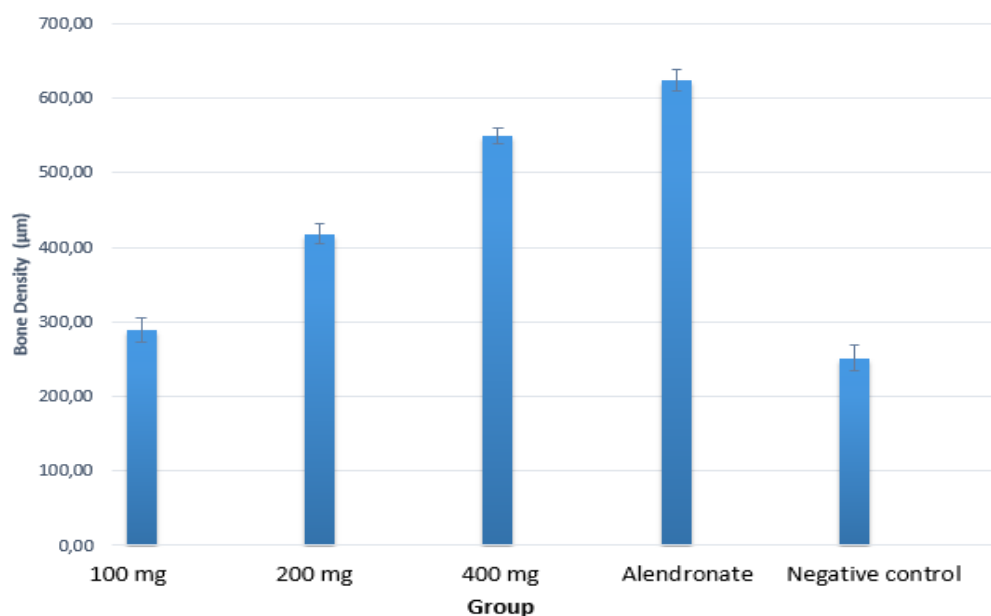


Figure 2. Bone density values of each group.

Table I. Bone Improvement Percentage

Groups	Bone Improvement Percentage
<i>C. cainito</i> ethanol extract dose 100 mg/kgBW mice/day	14.92 %
<i>C. cainito</i> ethanol extract dose 200 mg/kgBW mice/day	66.22 %
<i>C. cainito</i> ethanol extract dose 400 mg/kgBW mice/day	118.66 %
Positive Control (Alendronate 1.3 mg/kgBW mice/day)	147.93 %

Dexamethasone is one of the synthetic corticosteroids. By treatment this medicine for four weeks for mice, is equal to 3-4 years treatment for humans (Brunton *et al.*, 2005; Noor, 2014). The use of corticosteroid group compounds for a long-term period (3-6 months, or more) can cause the inhibition of bone formation process (Kemenkes RI, 2015). The use of glucocorticoid for long term also can reduce the level of estrogen and progesterone hormone, while the deficiency of estrogen is considered as one factor that causes the imbalance of bone remodeling process and has a role in increasing bone resorption process (Dipiro *et al.*, 2008). Figure 2 shows the average result of trabecular vertebrae bone density for each group, whereas (Table I) shows percentage of bone density improvement of trabecular vertebrae compared to negative control.

Homogeneity test of mice's bone density with  $p > 0.05$  showed that average data of bone density in the experimental group had a similar variant (homogenous). Normality test of average data of mice's bone density in each group with  $p > 0.05$ , showed that the data was normally distributed. Therefore, it fulfilled the parametric statistics test qualification of one-way ANOVA.

In one-way ANOVA statistical test, the significance level was  $p < 0.05$ . It showed that there was a minimal difference between a pair of the experimental group. To know the significant difference of experimental group, post hoc test was done using the LSD method. The LSD test result showed the significant difference between the bone density value of the treatment group with the dosage of 200 and 400 mg/kgBW mice/day compare to the negative control group with p-value 0.011 and 0.001. It showed that 70% ethanol extract of *C. cainito* leaves in that dosage could increase bone density. While for the extract with dosage of 100 mg/kgBW mice/day with p-value = 0.277 could not increase the bone density, because there was no significant difference with the negative control group. LSD test result showed the significant difference between two treatment groups 100 mg/kgBW mice/day and 200 mg/kgBW mice/day with alendronate group with p value each is 0.000 and 0.007. Meanwhile, for extract with the dose of 400 mg/kgBW mice/day showed that 70% ethanol extract of *C. cainito* leaves on that dosage did not have significant difference compare to alendronate group with p value 0.139.

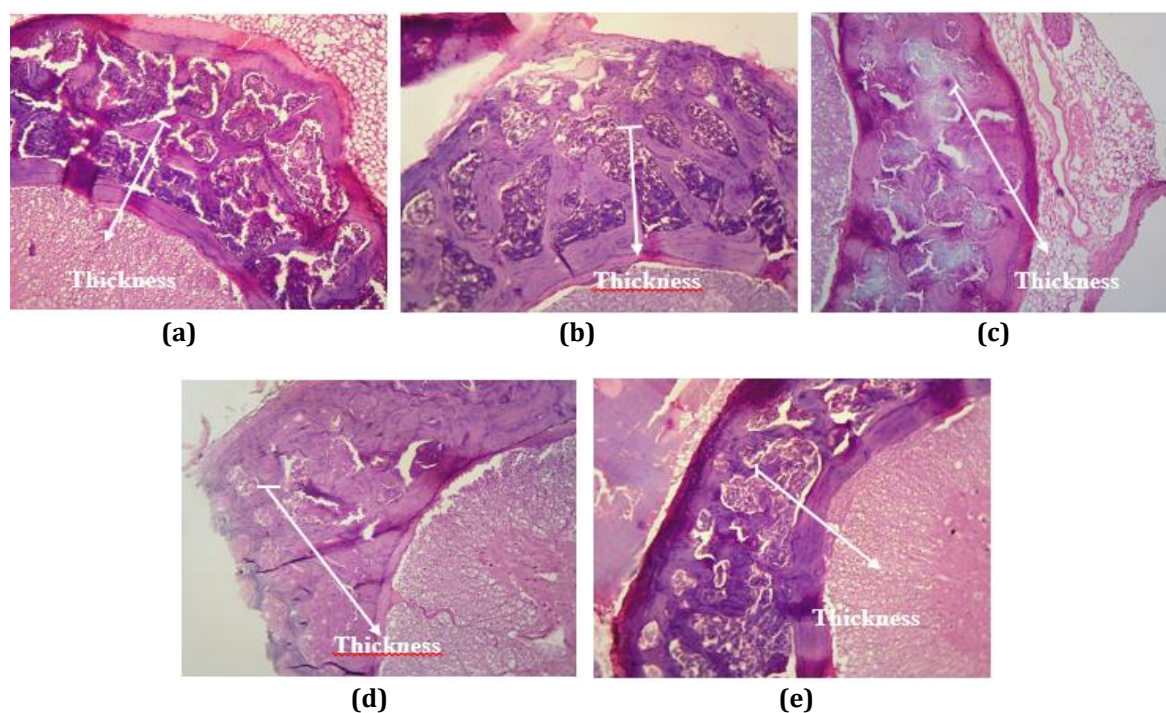


Figure 3. Histopathology of female mice's vertebrae trabecular bone; (a) Extract of *C. cainito* leaves 100 mg; (b) Extract of *C. cainito* leaves 200 mg; (c) Extract of *C. cainito* leaves 400 mg; (d) Alendronate; and (e) Negative Control

The result showed the tendency of improvement of trabecular vertebrae bone density value by giving 70% ethanol extract of *C. cainito* leaves. According to LSD test result, it was found that 70% ethanol extract of *C. cainito* leaves with dosage of 100 mg/kgBW mice/day did not have pharmacology effect. Dosage of 200 mg/kgBW mice/day could give pharmacology effect by increasing bone density, and for the dosage of 400 mg/kgBW mice/day had activity level that was almost equal with alendronate in increasing bone density.

The histopathology check was done and showed in Figure 3. The cancellous bone (x) and solid bone (y) were shown in different colors. There were alpha and beta estrogen receptor (ER $\alpha$  and ER $\beta$ ) expressions in bone, which had a role in maintaining homeostasis of bone density. In the situation of estrogen deficiency, which was caused by dexamethasone induction, there was no estrogen binding with ER. Therefore, it caused pro-osteoclastogenesis cytokines such as Tumor Necrosis Factor-Alpha (TNF- $\alpha$ ), Interleukin-1 (IL-1) and Interleukin-6 (IL-6) produced in high number. The production of this cytokines would induce preosteoclast differentiation to mature osteoclast, and would occur massive bone resorption process. Moreover, the increase of

cytokines also would inhibit preosteoblast differentiation to mature osteoblast, which then headed to the inhibition of bone formation process.

Insulin-like Growth Factor-1 (IGF-1) and Insulin-like Growth Factor-2 (IGF-2) in high number. The decreasing of growth factor could increase osteoclastogenesis and inhibit osteoblastogenesis, so that the osteoporosis tended to occur (Kawiyana, 2009; Kini and Nandeesh, 2012; Meeta, 2013).

In this research, the increasing of trabecular vertebrae bone density for female mice could occur because of phytoestrogens content in 70% ethanol extract of *C. cainito* leaves. The phytoestrogens compound were bind to ER in cell nucleus and reserved the situation that happened in estrogen deficiency (Yang *et al.*, 2012). Phytoestrogen binding with ER caused the decreasing of osteoclastogenesis and bone resorption, as well as the increasing of osteoblastogenesis and bone formation (Laswati *et al.*, 2015).

## CONCLUSION

The 70% ethanol extract of *C. cainito* leaves have activity in increasing trabecular vertebrae bone density for female mice with optimum dose of 400 mg/kgBW mice/day. This activity can occur because phytoestrogens content in 70% ethanol

extract of *C. cainito* leaves, that can replace the function of estrogen in its bond with ER.

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