

ACTIVITY OF 96% ETHANOL EXTRACT OF *CHRYSOPHYLLUM CAINITO* L. IN INCREASING VERTEBRAE TRABECULAR OSTEOBLAST CELL NUMBER IN MALE MICEBURHAN MA'ARIF^{1*}, AGNIS PONDINEKARIA ADITAMA²¹Department of Pharmacy, Faculty of Medical and Health Science, Maulana Malik Ibrahim State Islamic University, Malang, East Java, Indonesia.²Department of Pharmacy, Pharmacy Academy of Jember, Jember, East Java, Indonesia. Email: burhan.maarif@farmasi.uin-malang.ac.id

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ABSTRACT

Objectives: This study was conducted to analyze the anti-osteoporosis effect of 96% ethanol extract from *Chrysophyllum cainito* L. leaves, which suspected to contain phytoestrogens, in increasing the osteoblast cell number in trabecular vertebra bone of dexamethasone-induced male mice.

Materials and Methods: The 96% ethanol extract of *C. cainito* leaves was given to male mice with dose of 2, 4, 8, and 16 mg/20 g BB mice/day after being induced orally with dexamethasone dose of 0.0029 mg/20 g BB mice/day. The positive control used was 0.026 ml/20 g BB mice/day alendronate. After 4 weeks, the increasing of osteoblast cell number in trabecular vertebrae bone of male mice was observed using an optical microscope with 100× zoom in after histomorphometry and hematoxylin-eosin staining methods.

Results: The result showed the significant increasing of osteoblast number in trabecular vertebrae bone of male mice in all groups after being given treatment using 96% ethanol extract of *C. cainito*, with an effective dose (ED₅₀) value of 9.5 mg/20 g BB mice/day. This increase is suspected due to the phytoestrogens content, which can also act as phytotestosterone in 96% ethanol extract of *C. cainito*.

Conclusion: This study concluded that 96% ethanol extract of *C. cainito* has an activity in increasing osteoblast cell number in trabecular vertebra bone of dexamethasone-induced male mice, with an ED₅₀ value of 9.5 mg/20 g BB mice/day.

Keywords: *Chrysophyllum cainito* L., Phytoestrogens, Osteoporosis.

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INTRODUCTION

Osteoporosis is a condition with the degradation of bone mass along with microarchitecture damage of bone which causes the fracture risk [1,2]. Osteoporosis usually occurs in postmenopausal women, which is suffering from estrogen deficiency [3].

Giving hormone replacement therapy (HRT) is known to be a treatment that is often given to patients with estrogen deficiency. This is because HRT can replace the function of estrogen in maintaining homeostasis function of body organs including preventing osteoporosis [3,4]. However, in some studies, it is known that long-term administration of HRT may lead to potential side effects such as coronary events, venous thromboembolism, stroke, breast cancer, and dementia [5,6].

Phytoestrogens are a group of compounds derived from plants that have estrogen-like structures or can replace the function of estrogen in its bond either with estrogen receptors ([ERs]-dependent pathway) or not (ER-independent pathway) [7,8]. Besides being easy to obtain and having no side effects, the phytoestrogens are also reported to have activity for the treatment of estrogen deficiency [9-11], thereby providing an alternative treatment for potent osteoporosis [12].

Chrysophyllum cainito L. is a plant that is known to contain the phytoestrogens. This plant is growing a lot in East Java, Indonesia, and usually used to cure diabetes mellitus and rheumatic joints [13]. However, *C. cainito* has not been widely studied yet. In the previous studies, it was known that *C. cainito* leaves contain compounds such as alkaloid, phenol, flavonoid, triterpenoid, and sterol [14]. Either isoflavones which are included in flavonoid compounds or sterols can be estimated as phytoestrogens compounds due to their similar structure with 17 β -estradiol [15].

Steroid sex hormone such as estrogen and testosterone plays an important role for growth and maintains bone density either in women or man. There is a significant relationship between bone mineral density and estrogen levels in women or testosterone in man [16]. Hence, there is a possibility that phytoestrogens in *C. cainito* can also act as phytotestosterone.

The aims of this study were to analyze the anti-osteoporosis effect of 96% ethanol extract from *C. cainito* L. leaves, in increasing the osteoblast cell number in trabecular vertebra bone of dexamethasone-induced male mice (*Mus musculus*), osteoblast in trabecular vertebrae bone should be counted after giving 96% ethanol extract of *C. cainito* leaves as samples. The increasing of osteoblast cell number in trabecular vertebrae bone of male mice was observed using histomorphometry and hematoxylin-eosin staining methods.

MATERIALS AND METHODS**Plant material**

C. cainito leaves were taken and identified in Unit Pelaksana Teknis Materia Medica, Batu, Indonesia, on October 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 leaves powder.

Drugs and chemicals

The 96% ethanol, dexamethasone, alendronate, CMC Na 0.5%, and chloroform were obtained from Phytochemistry Laboratory in Pharmacy Department, Faculty of Medicine and Health Science, Maulana Malik Ibrahim State Islamic University, Malang. The 10% formalin, 10% formic acid, 70% alcohol, 3% nitrate acid, acetone, xylol, liquid paraffin, glycerin, water ammonia, and hematoxylin and eosin dye were obtained from Parasitology Laboratory, Faculty of Medicine, Brawijaya University, Malang.

Extract preparation

The dry powder of *C. cainito* leaves was extracted with 96% ethanol using ultrasonic assisted extraction method. This process was repeated, collecting all the supernatants, which were finally evaporated in a rotary evaporator to get 96% ethanol extract. The extract prepared to produce extract suspension in water with the dose of 2, 4, 8 and 16 mg/20 g BB mice/day.

Bone specimen preparation

Male mice were induced with dexamethasone of 0.0029 mg/20 g BB mice/day orally. Then, each group was treated with 2, 4, 8, 16 mg/20 g BB mice/day samples, and 0.026 ml/20 g BB mice/day alendronate. The treatment was given orally in 4 weeks. The next step was doing mice surgery to take the trabecular vertebrae bone. The vertebrae bone was cut in segment 2-7 and put inside the small container with 10% formalin for temporary storage. Trabecular vertebrae bone then inserted in decalcification solution (7.0 g aluminum chloride, 8.5 g chloride acid, and 5.0 ml formic acid in 100 ml water). Next step was neutralization using sodium sulfate 2% for 24 h. Vertebrae bone then was washed using water flows for 12 h and rinsed with 70% alcohol. The bone then blocked using paraffin and cut using microtome. Next step was hydration using 70% alcohol and hematoxylin as the main coloring. The bone then inserted in 1% acid alcohol and water ammonia and dropped by comparator coloration 1% eosin. The bone was dehydrated using 70% alcohol. The final step was clearing using xylol and mounting by placing the bone on object glass. The observation was done using an Olympus Optical Microscope with 100x zoom in every slide.

Data analysis

The research result was analyzed using one-way ANOVA. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Extraction of 30 g *C. cainito* leaves with 96% ethanol produces extracts with a yield value of 7.82%. Osteoporosis condition in mice was created using dexamethasone induction with the dose of 0.0029 mg/20 g BB mice/day. Mice with osteoporosis condition could be differentiated by looking at their bent spine (kyphotic), as shown in Fig. 1.

Dexamethasone is one glucocorticoid drug. Treating mice with glucocorticoid for 4 weeks are equal to 3-4 years of treatment for humans [16]. Glucocorticoid is an estrogen agonist which has the same steroids structure and can bind the ER and cause estrogen deficiency by the production of mRNA sulfotransferase [17]. Therefore, using

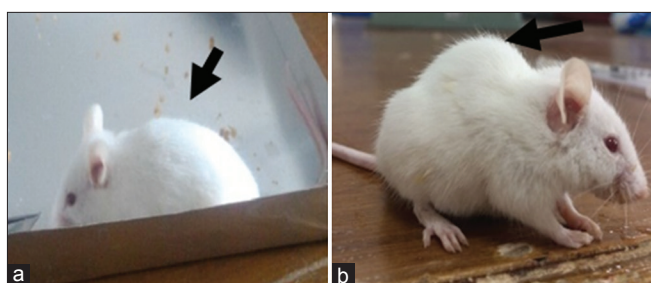


Fig. 1: (a) Normal mice and (b) mice with osteoporosis

glucocorticoids for long-term therapy can cause the inhibition of bone formation process and cause osteoporosis [17,18].

The retrieval of data was obtained from the osteoblast cell number calculation in trabecular vertebrae bone of male mice. All the obtained data were normally distributed and homogenous. Table 1 and Fig. 2 show the average result of total osteoblast cell number for each test group.

In one-way ANOVA statistical test, the statistical significance level was $p < 0.05$. It showed that there was a significant difference in the number of osteoblast cells between each group. To know the significant difference of the experimental group, *post hoc* test was done using LSD method. The LSD test result also showed that the significant difference between osteoblast numbers of all dosage treatment groups compares to the negative control group. It showed that 96% ethanol extract of *C. cainito* leaves in those dosages could increase osteoblast number. Besides that, LSD test result also showed the significant difference between 16 mg/20 g BB mice/day dose with alendronate group with value of $p = 0.005$. Meanwhile, for extract with the dose of 2, 4, and 8 mg/20 g BB mice/day had $p = 0.559, 0.895,$ and 0.943 showed that 96% ethanol extract of *C. cainito* leaves on those dosages did not have significant difference compare to alendronate group.

The result showed that there is a tendency of increasing osteoblast number by giving 96% ethanol *C. cainito* leaves extract. According to LSD test result, it was found that 96% ethanol extract of *C. cainito* leaves with all dosage test has pharmacology effect. The dose of 8 mg/20 g BB mice/day had activity level that was almost equal with alendronate, and dose of 16 mg has better activity than alendronate in increasing the number of osteoblast cell in male mice. The histopathology of trabecular vertebrae bone of male mice can be seen in Fig. 3.

This activity was suspected due to phytoestrogens content in 96% ethanol extract of *C. cainito* leaves that also act as phytotestosterone. It probably can happen because testosterone has the same steroid core structure as estrogen.

Long-term administration of glucocorticoids has been shown to cause hypogonadism which results in decreased testosterone levels. Whereas if the testosterone level was decreased, the bone formation will

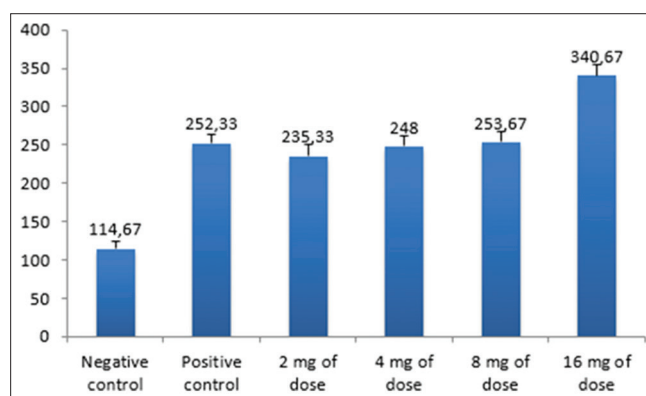


Fig. 2: Osteoblast number of each group

Table 1: Osteoblast number of each group

Groups	Average osteoblast number/five field of view \pm SD
Positive control	252.33 \pm 10.39
Negative control	114.67 \pm 10.07
<i>C. cainito</i> ethanol extract dose of 2 mg/20 g BB mice/day	235.33 \pm 15.88
<i>C. cainito</i> ethanol extract dose of 4 mg/20 g BB mice/day	248 \pm 14.47
<i>C. cainito</i> ethanol extract dose of 8 mg/20 g BB mice/day	253.67 \pm 13.51
<i>C. cainito</i> ethanol extract dose of 16 mg/20 g BB mice/day	340.67 \pm 14.52

C. cainito: *Chrysophyllum cainito*

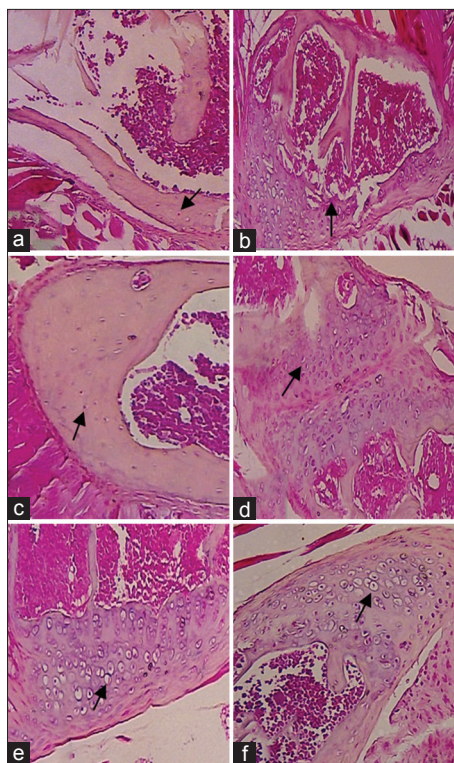


Fig. 3: Histopathology of trabecular vertebrae bone of male mice: (a) Negative control, (b) positive control, (c) dose of 2 mg, (d) dose of 4 mg, (e) dose of 8 mg, (f) dose of 16 mg

automatically be disrupted so is the case with estrogen. This is because testosterone plays a role in binding directly to androgen receptors for bone growth and maintaining bone density [19]. That is why with the administration of phytotestosterone contained in 96% ethanol extract of *C. cainito* leaves can restore bone homeostasis.

CONCLUSION

The 96% ethanol extract of *C. cainito* L. leaves had an activity to increase osteoblast cell number in trabecular vertebrae bone for male mice with ED_{50} is 9.5 mg/20 g BB mice/day.

AUTHORS' CONTRIBUTIONS

The authors declare that this work was done by the author named in this article.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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