

**Effect of *Chrysophyllum cainito* L. Leaves on Bone Formation *In Vivo* and *In Silico***Pramudita Riwanti<sup>1</sup>, Miftah S. Arifin<sup>2</sup>, Faisal A. Muslikh<sup>3</sup>, Dilla Amalia<sup>3</sup>, Iffatul Abada<sup>3</sup>, Agnis P. Aditama<sup>4</sup>, Burhan Ma'arif<sup>3\*</sup><sup>1</sup>Department of Pharmacy, Faculty of Medicine, Hang Tuah University, Surabaya 60111, Indonesia<sup>2</sup>Master Program in Biomedical Sciences, Faculty of Medicine, Brawijaya University, Malang 65145, Indonesia<sup>3</sup>Department of Pharmacy, Faculty Medical and Health Science, Maulana Malik Ibrahim State Islamic University, Malang 65151, Indonesia<sup>4</sup>Department of Pharmacy, Pharmacy Academy of Jember, Jember 68125, Indonesia

## ARTICLE INFO

## Article history:

Received 30 October 2020

Revised 25 January 2021

Accepted 16 February 2021

Published online 01 March 2021

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## ABSTRACT

Phytoestrogens are compounds that have estrogen-like functions and have the ability to overcome osteoporosis. *Chrysophyllum cainito* L. is a plant that contains phytoestrogens. This study aimed to identify the activity of 96% ethanol extract of *C. cainito* leaves in increasing the bone density in trabecular vertebrae bone of dexamethasone-induced male mice. The prediction of phytoestrogen compounds that have the bone induction effect was done using *in silico* study. A 96% ethanol extract of *C. cainito* leaves was given at 0.1; 0.2; 0.4; and 0.8 mg/g/day after being induced with dexamethasone. The increase in bone density was observed after histomorphometry. The *in silico* study was done using metabolite profiling data from a previous study as a sample. The result showed the increase of bone density in all groups, with the best dose at 0.1 mg/g/day with bone density value  $266.65 \pm 1.38 \mu\text{m}$ , and ED<sub>50</sub> value of 95.4 mg/g/day. This effect was suspected to be due to phytoestrogens content and supported by the data from *in silico* study, which showed that eight compounds were predicted to have similar activities to 17 $\beta$ -estradiol. It was also found that phytoestrogens can influence bone formation in male mice because of their steroid core structure similarities with phytoestosterone.

**Keywords:** Antiosteoporosis, Molecular docking, Natural product, Phytoestrogens.

## Introduction

Osteoporosis is a condition where bone mass degradation occurs. The condition causes bone microarchitecture damage, which can increase the risk of fractures.<sup>1,2,3</sup> Prevalence of osteoporosis reached 36 million by 2013.<sup>4</sup> Osteoporosis is usually experienced by postmenopausal women, who are suffering from estrogen deficiency and affects the balance of the bone remodelling process.<sup>5,6</sup> The treatment considered suitable for patients with estrogen deficiency induced-osteoporosis was hormone replacement therapy (HRT). However, long-term therapy of HRT may lead to several side effects and higher costs, such as stroke, breast cancer, coronary events, dementia, and venous thromboembolism.<sup>6-10</sup>

Phytoestrogens are plant compounds that have estrogen-like functions or structures. It can replace the estrogen in its bond with estrogen receptors (ERs) as phytoestrogens can substitute the receptors.<sup>11,12</sup> Phytoestrogens are known to have no side effect and have acted for the treatment of estrogen deficiency induced-diseases,<sup>13,14,15</sup> such as osteoporosis.<sup>16</sup>

Plants are a source of many potent and powerful drugs. Plants with healing properties are medicinal plants or herbs.<sup>16,17</sup> Herbal medicines from plants represent an important source of medical compounds and have been observed as therapeutic agents able to cure several types of health problems.<sup>18-20</sup> *Chrysophyllum cainito* L. (*C. cainito*) is a plant suspected to contain phytoestrogens. This plant can be found easily in

East Java and has several medical functions.<sup>21-23</sup> *C. cainito* leaves contain compounds such as phenol, alkaloid, isoflavonoid, sterol, and triterpenoid.<sup>24</sup> Both isoflavones or sterols are known as phytoestrogens due to their similar structure with 17 $\beta$ -estradiol.<sup>25</sup>

Sex hormones, such as estrogen and testosterone have crucial roles in maintaining bone homeostasis, both in women and men. There is a relationship between bone density with estrogen concentration in women or testosterone in men.<sup>26</sup> There is a possibility that phytoestrogens in *C. cainito* can also act as phytoestosterone.

This study aimed to identify the activity of 96% ethanol extract of *C. cainito* leaves in increasing the bone density of trabecular vertebra bone of dexamethasone-induced male mice (*Mus musculus*). The analysis was to predict the phytoestrogen compounds using *in silico* study and then analyze with molecular docking, one of the most frequently used methods in structure-based drug design. This method can predict the binding-conformation of small molecular ligands.<sup>27,28</sup> The active site of the protein was firstly identified as the binding site. It uses interacting molecules to decide the signal-transduction as agonism or antagonism.<sup>29,30</sup> This was conducted with a view to developing new drugs from natural ingredients. *C. cainito* has a potential as an anti-osteoporosis agent in postmenopausal women with minimum side effects.

## Materials and Methods

## Plant materials

*C. cainito* leaves were obtained and identified in UPT Materia Medica, Batu, Indonesia, in October 2017 with specimen number 1b-2b-3b-4b-5b-6b-7b-8b-9b-10b-11b-12b-13b-14a-15a-109b-119b-120a-121b-124b-125a-126b-127a. The leaves were dried and ground to produce dry powder of *C. cainito* leaves.

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**Citation:** Riwanti P, Arifin MS, Muslikh FA, Amalia D, Abada I, Aditama AP, Ma'arif B. Effect of *Chrysophyllum cainito* L. Leaves on Bone Formation *In Vivo* and *In Silico*. Trop J Nat Prod Res. 2021; 5(2):260-264. [doi.org/10.26538/tjnpr/v5i2.8](https://doi.org/10.26538/tjnpr/v5i2.8)

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

### Experimental animals

*Mus musculus* (20-25 g) were from the Faculty of Animal Medicine, Universitas Airlangga, Surabaya, Indonesia. Ethical clearance under the care and use of the laboratory animals is from the Ethical Clearance Committee, Faculty of Medical and Health Sciences, Maulana Malik Ibrahim State Islamic University, Malang. The clearance number was 020/EC/KEPK-FKIK/2018. The protocols are in line with the standard laboratory conditions and practices.

### Chemical

Ethanol (96%) and acetone were from Merck. The other chemicals, like formic acid (10%), formalin (10%), nitrate acid (3%), glycerin, xylol, liquid paraffin, water, ammonia, decalcification solution, hematoxylin, and eosin dye were from Sigma Aldrich.

### Extraction

The dry powder of *C. cainito* leaves (30 g) was extracted with 96% ethanol (500 mL) using ultrasonic-assisted extraction methods (Sonica 5300P S3). This process was repeated, collecting all the supernatants, which were finally evaporated in a rotary evaporator (HEIDOLPH Hei-VAP G3) to obtain 96% ethanol extract of *C. cainito* leaves. The extract was prepared to produce suspension in water at dose of 0.1; 0.2; 0.4; and 0.8 mg/g/day.

### In Vivo Study

In vivo study was carried out in the Biomedical Laboratory, Faculty of Medical and Health Sciences, Maulana Malik Ibrahim State Islamic University, Malang. The mice were acclimatized for seven days prior to treatment. They were maintained with water and feed before the experiment with standard conditions (temperature: 25-34°C). The mice were induced orally with dexamethasone of 0.000145 mg/g/day. Each group was treated with 0.1; 0.2; 0.4; and 0.8 mg/g/day samples, and 0.0013 mg/g/day alendronate as positive control, all given orally for four weeks. After that, the bone was cut in segments 2-7 and put inside a container with 10% formalin, then inserted in decalcification solution and washed using 2% sodium sulfate. Hydration was done using 70% alcohol. The bone was then blocked with paraffin and cut with a microtome. The next step was coloring the bone with both hematoxylin and 1% eosin. The bone was then washed using xylol and prepared on an object-glass. The observation was done using Optical Microscope at 100x zoom.

### In Silico Study

The samples for *in silico* study were compounds from metabolite profiling result of 96% ethanol extract of *C. cainito* leaves using UPLC-QToF-MS/MS from our previous study.<sup>31</sup> Receptor structure of ER $\beta$  used in this research was from <http://www.rcsb.org> with code 3OLS. Preparation was to separate ligand 17 $\beta$ -estradiol from the 3OLSoprotein using Biovia Discovery Studio Visualizer 2016. The sample preparation was to predict its physicochemical properties using SwissADME Webtool. The energy optimization of ligand 17 $\beta$ -estradiol and sample structure was from Avogadro 1.90. Molecular docking was done using PyRx 0.8 software and the AutoDock Vina method. The value of binding affinity, pharmacophore distance, and type of bound amino acids from the sample analysis was to determine the phytoestrogen compounds. The results were agonists with 17 $\beta$ -estradiol using Biovia Discovery Studio Visualizer 2016.

### Statistical analysis

The result of the *in vivo* study was analyzed using one-way ANOVA. Differences were considered significant at  $p < 0.05$ . Post hoc test was done using LSD test while the ED<sub>50</sub> value was calculated by probit analysis under homogeneous conditions and normal distribution.

## Results and Discussion

The extraction of 500 g of *C. cainito* leaves produced 25.55 g of extract (yield value of 5.71%). Osteoporosis in mice was to create dexamethasone induction. Mice with osteoporosis were differentiated

by kyphotic at their spine. Analysis of bone formation used trabecular vertebrae bone because this part of the bone is most often affected when an imbalance occurs in the bone remodeling process.

Dexamethasone is one of the glucocorticoids. Treating mice with glucocorticoid for four weeks is equal to three to four years of treatment for humans.<sup>32</sup> Glucocorticoids have similar structure with 17 $\beta$ -estradiol. Glucocorticoids can bind the ERs and cause estrogen deficiency by the induction of mRNA sulfotransferase.<sup>33</sup> Therefore, using glucocorticoids for long-term treatment can cause the inhibition of the bone formation process and cause osteoporosis.<sup>33,34</sup>

Figure 1 shows the histological observation of male mice trabecular vertebrae bone density in each group. The Motic Image Plus 3.0 software was used to measure bone density in the metaphysical section. Metaphysics plays an active role in bone growth and lies beneath the epiphyses. It can influence the formation of a compact bone structure or bone cavity.<sup>35</sup>

The data from histological observations of the trabecular vertebrae bone of mice show the average result of bone density for each test group [Table 1 and Figure 2]. In the one-way ANOVA test, the significance level used was  $p < 0.05$ . A LSD *post hoc* test was employed to know the significant difference of the experimental group. The LSD test result showed a significant difference between the bone density of all dose-treatment groups. The results were compared to the negative control group. Based on these data, 96% ethanol extract of *C. cainito* leaves at all doses tested have pharmacological effect. The LSD test result also showed significant difference between 0.1; 0.2; 0.4; and 0.8 mg/g/day dose and the alendronate group, which showed that dose 0.1; 0.2; 0.4; and 0.8 mg/g/day have a better activity in increasing bone density than alendronate. The dose of 0.1 mg/g/day had the best activity in increasing the male mice bone density. The ED<sub>50</sub> value was calculated using probit analysis and ED<sub>50</sub> of 95.4 mg/g/day was obtained.

However, the increasing dose of treatment is not in line with a bone density increase (Figure 2). A non-monotonic dose response (NMDR) effect was observed. The NMDR with varying slope values was indicated at several points in the given dose range.<sup>36</sup> This phenomenon often occurs in research with hormone or hormone substituents as samples, in this case, the phytoestrogen compounds in the 96% ethanol extract of *C. cainito* leaves. Differences in the level of affinity between hormones or hormone replacement samples with receptors at both target receptors and non-target receptors in cells will cause difficulties in predicting responses that will arise with increasing doses.<sup>37</sup>

Phytoestrogens will bind to ER in the nucleus of bone cells to restore the estrogen deficiency.<sup>15</sup> It will cause a decrease and increase of osteoclastogenesis and there will be an increase in bone formation and bone density.<sup>38,39</sup>

From this study, it was found that phytoestrogens can influence male mice bone formation because of steroid core structure similarities between estrogen and testosterone in male animals. Phytoestrogens can bind with androgen receptor (AR). AR co-activator generated androgenic effects of phytoestrogens. The correlation between phytoestrogens and androgen activity happened because of the ligand-receptor-cofactor contact. Hence, phytoestrogens might be able to act as phytotestosterone.<sup>40</sup> Long-term treatment of glucocorticoids has proven to cause hypogonadism if the testosterone level decreases. Then, the bone formation is disrupted in line with estrogen. Testosterone plays a role in binding directly to androgen receptors for bone growth and maintaining bone density.<sup>41</sup>

*In silico* study is a fast and inexpensive approach to predict phytoestrogen compounds found in 96% ethanol extract of *C. cainito* leaves in inducing bone formation process by increasing bone density.<sup>42</sup> *In silico* study sample was metabolite profiling of 96% ethanol extract of *C. cainito* leaves with UPLC-QToF-MS/MS, as obtained from previous studies.<sup>43</sup>

The screening of 41 compounds from the metabolite profiling analysis was done with SwissADME Webtool to know the physicochemical properties.<sup>44</sup> The compounds analysis program was PyRx 0.8 software with molecular docking and AutoDock Vina as a docking simulator.<sup>45</sup> From the redocking test, the RMSD value was  $< 2 \text{ \AA}$ , so that the docking protocol was suitable in docking the samples. Eight

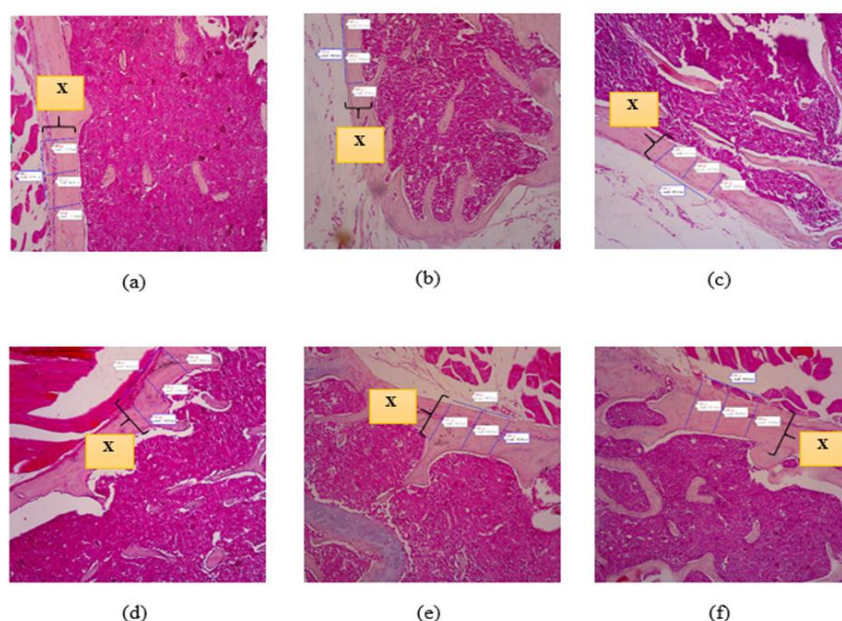
compounds were phytoestrogens or 17 $\beta$ -estradiol agonists from the analysis results with Discovery Studio Visualizer 2016 (Table 2). Similar binding affinity values, pharmacophore distances, and type of bound amino acids showed identical activity.<sup>46</sup> Therefore, binding affinity, pharmacophore distances, and types of bound amino acids in the sample were compared with 17 $\beta$ -estradiol's and can be seen in Table 3 and Figure 3. The compound must interact with its 475 amino acid residues and interact either with Arg 346 or Glu 305 to act as a 17 $\beta$ -estradiol agonist, both of them with hydrogen bonds. The absence of interaction between the compound and its 475 residues will cause the blend to act as a 17 $\beta$ -estradiol antagonist.<sup>47</sup> However, the activity in increasing bone density was the effect of a single compound alone. Due to the multi-compounds content in 90% ethanol extract of *C. cainito* leaves, the activity arose because of the synergistic effect of several blends at once in the form of a multi-target receptor mechanism.

**Table 1:** Trabecular vertebrae bone density value of male mice after administration of 96% ethanol extract of *C. cainito* leaves.

Groups	Bone Density ( $\mu\text{m}$ ) $\pm$ SD
Negative Control	75.08 $\pm$ 2.32
0.1 mg/gBW mice/day	266.65 $\pm$ 1.38
0.2 mg/gBW mice/day	179.1 $\pm$ 2.81
0.4 mg/gBW mice/day	214.36 $\pm$ 4.27
0.8 mg/gBW mice/day	224.6 $\pm$ 3.68
Alendronate	178.29 $\pm$ 2.37

**Table 2:** Prediction of agonist compounds in 96% ethanol extract of *C. cainito* leaves against 3OLS protein

No	Compounds	Binding Affinity	Pharmacophore Distance	Amino Acid	Type of Bond
1	11-Aminoundecanoic acid	-5.8	10.637	His 475; Glu 305; Arg 346	Hydrogen; Unfavorable
2	Megalanthonine	-7.3	9.397	His 475; Glu 305	Hydrogen; Carbon
3	epi-jasmonic acid	-6.7	9.125	His 475; Glu 305	Hydrogen; Alkyl
4	1-Amino-3-(hexadecyloxy)-2-propanol	-5.7	10.203	His 475; Glu 305; Arg 346	Hydrogen; Alkyl; Unfavorable
5	Terephthalohydrazide	-6.8	8.865	His 475; Glu 305; Arg 346	Attractive Charge; Unfavorable
6	1-[(6,7-Dimethyl-2-oxo-1,2-dihydro-3-quinolinyl)methyl]-1,3-bis[3-(4-morpholinyl)propyl]thiourea	3.5	9.903	His 475; Glu 305	Hydrogen; Unfavorable
7	IsoSildenafil	9.2	10.833	His 475; Glu 305	Hydrogen
8	N-[(3R,4S)-9-[(5-Chloro-1,3-dimethyl-1H-pyrazol-4-yl)methyl]-3-hydroxy-4-methyl-1-oxa-9-azaspiro[5.5]undec-4-yl]-2-methoxyacetamide	19.7	10.792	His 475; Glu 305; Arg 346	Hydrogen; Unfavorable

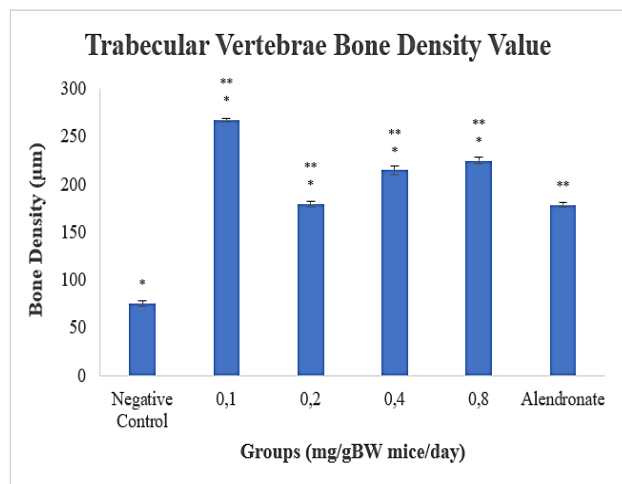


**Figure 1:** Histology of male mice trabecular vertebrae bone density: (a) Positive control (Alendronate), (b) Negative control, (c) 0.1 mg/g/day, (d) 0.2 mg/g/day, (e) 0.4 mg/g/day, (f) 0.8 mg/g/day, and X is the epiphyse part which is measured in trabecular vertebrae bone.



**Table 3:** Analysis of 17 $\beta$ -Estradiol activity on the 3OLS protein

Sample	Binding Affinity	Pharmacophore Distance	Amino Acid	Type of Bond
17 $\beta$ -Estradiol	-10.5	10,862 Å	His 475	Hydrogen
			Glu 305	Hydrogen
			Arg 346	Hydrogen



**Figure 2:** Trabecular vertebrae bone density value of mice after the administration of 96% ethanol extract of *C. cainito* with variation dose. Each value is expressed as the mean  $\pm$  SD. Significant differences in compared with negative control (\*), and positive control (alendronate) (\*\*) at  $p < 0.05$

phytoestrogens content in *C. cainito* leaves. The data from the *in silico* study showed eight compounds predicted to have similar activities to 17 $\beta$ -estradiol. It was also found that phytoestrogens can influence bone formation in male mice because of the steroid core structure similarities with phytotestosterone.

### Conflict of interest

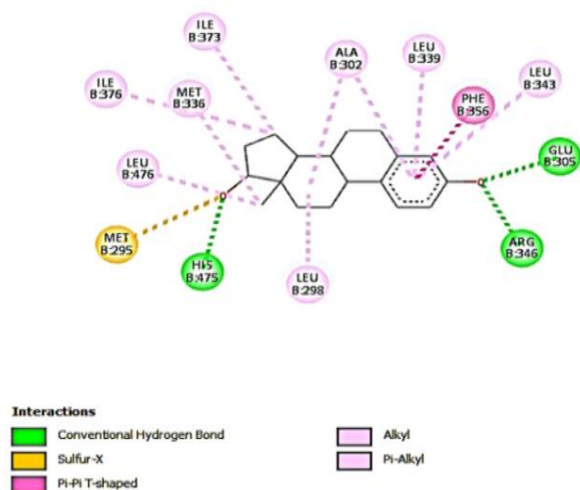
The authors declare no conflict of interest.

### Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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**Figure 3:** The bond between 17 $\beta$ -estradiol to amino acids in the 3OLS protein.

### Conclusion

The 96% ethanol extract of *C. cainito* leaves increased the bone density of trabecular vertebrae bone of male mice at all tested doses with the best dose at 0.1 mg/g/day (bone density value 266.65  $\pm$  1.38  $\mu$ m) and ED<sub>50</sub> value of 95.4 mg/g/day. This increase is due to the

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