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Original Article

The Effect of Herbal Tablets from *Chrysophyllum cainito* L. Leaves Extract on Increasing Osteoblast Cell Number and Bone Mass Density in Wistar Rats

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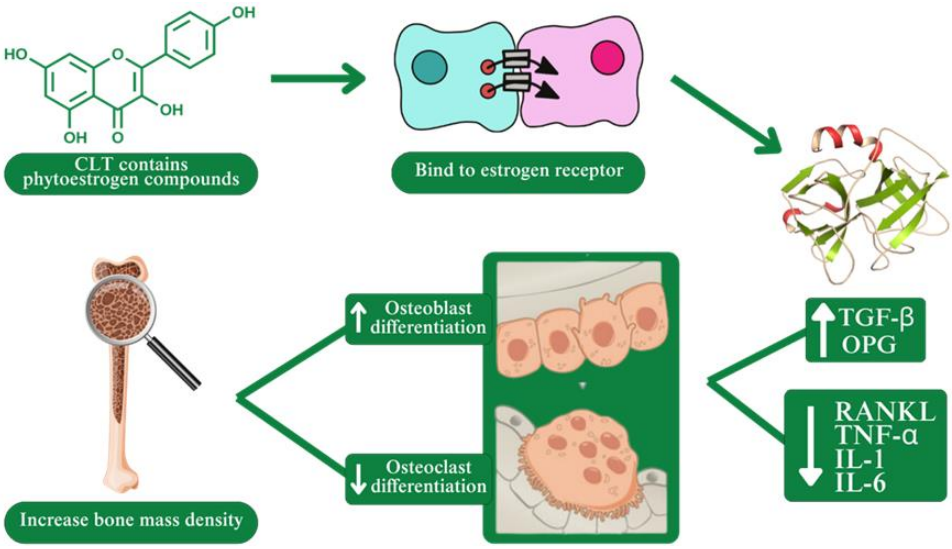
ABSTRACT

The presence of bone metabolic imbalances in bone remodelling is the cause of osteoporosis. Phytoestrogens, natural compounds found in plants with estrogen-like properties, can be obtained from the leaves of *Chrysophyllum cainito* L, and are capable of replacing the role of estrogen in maintaining the regulation of bone remodelling, which inhibits osteoporosis. The study aims to determine the activity of herbal tablets formulated from the ethanol extract of *C. cainito* leaves (CLT) in increasing osteoblast cell number and bone mass density in the femur and vertebrae trabecular bone of the osteoporosis-induced Wistar rat (*Rattus norvegicus*). The rats used were 35 female ovarectomized Wistar rats. After the rats underwent osteoporosis due to estrogen deficiency, alendronate (0.18 mg/200 g BW rats/day) was used as a positive control, whereas CLT was given at doses of 24.3, 48.6, and 97.2 mg/200g BW rats/day orally. This study used a 400x light microscope to look at the osteoblast cell number and bone mass density. The cells were coloured with hematoxylin and eosin. The results demonstrate that CLT in all doses can significantly increase both the osteoblast cell number and bone mass density in the trabecular femur and vertebrae, with an optimal dose of 48.6 mg/200 g BW rats/day. CLT has antiosteoporosis activity due to its phytoestrogen compounds, so it has the potential to be used as an antiosteoporosis alternative therapy.

GRAPHICAL ABSTRACT



Introduction

Osteoporosis (OP) is a disease characterized by reduced bone mass and changes in the microarchitecture of bone tissue caused by an imbalance in bone metabolism [1, 2]. Several studies state that the OP incidence in the world in 2021 will reach 18.3% with the number of OP sufferers reaching 49 million people, while in 2022 the increase will reach 19.7% [3, 4]. Osteoporosis predominantly manifests in postmenopausal women, primarily due to the decreased production of estrogen by the body [5]. Decreased estrogen and testosterone levels in the body have side effects in the bone remodelling process by inhibiting the formation of osteoblast cells and increasing osteoclast cell differentiation, resulting in increased production of pro-osteoclastogenesis cytokines such as Tumor Necrosis Factor-Alpha (TNF-α), Interleukin-1 (IL-1), and Interleukin-6 (IL-6), which cause excessive bone resorption. Apart from that, estrogen deficiency also causes a decrease in levels of tumour growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), and insulin-like growth factor-2 (IGF-2), which causes a balance in body density. Bones are disturbed, and OP can occur [6]. Hormone Replacement Therapy (HRT) is known to be a treatment that is often given to patients with estrogen deficiency because it can replace the function of estrogen in maintaining the homeostasis function of the body's organs, including preventing OP [7]. However, in several studies, it is known that long-term administration of HRT can cause potential side effects such as coronary events, venous thromboembolism, stroke, breast cancer, and dementia [8, 9]. The existence of various adverse side effects caused by HRT has encouraged research to find alternative treatments with minimal or even no side effects [10]. *Chrysophyllum cainito* L. is a plant that is known to contain phytoestrogen compounds. Phytoestrogens are compounds that come from plants and have a structure similar to estrogen so that they can replace the function of estrogen in binding to estrogen receptors, either through dependent pathways or independent pathways [11]. The absence of side effects on phytoestrogens makes it an alternative choice for treating OP caused by estrogen deficiency [12].

The content of secondary metabolite compounds found in *C. cainito* leaves, such as alkaloids, phenols, flavonoids, triterpenoids, and sterols [13]. The content of these compounds makes *C. cainito* leaves have great potential for use as an alternative treatment for OP through an estrogenic mechanism [14].

In this study, female ovarectomized Wistar rats (*Rattus norvegicus*) were used to test the anti-OP activity of tablets containing a 70% ethanol extract of *C. cainito* leaves (CLT). The increased osteoblast cell number and bone mass density in the trabecular femur and vertebrae were measured to determine its effect as an alternative anti-OP therapy.

Materials and Methods

Plants

The leaves of *C. cainito* L. were obtained in Batu, Indonesia, in June 2022 and identified at the UPT. Materia Medica Laboratory, Batu, Indonesia, with identification letter no. 067/1606/102.20/2023. The leaves were ground and dried to make a dry powder of *C. cainito* leaves.

Animals

Female Wistar rats (*Rattus norvegicus*) were obtained from the experimental animal laboratory at the BioScience Institute, Brawijaya University, Malang, Indonesia, with ethical clarification letter no. 136-KEP-UB-2023.

Tablet

CLT were obtained from PT. Agaricus Sido Makmur Sentosa, a pharmaceutical industry in Malang, Indonesia. The tablets weigh 900 mg, with the main content of each tablet equivalent to 150 mg of a 70% ethanol extract of *C. cainito* leaves. The composition of the tablet excipients is lactose, microcrystalline cellulose, sodium starch glycolate, hydrate silica, magnesium stearate, copovidine, and methylparaben.

Chemical

70% ethanol, tween 80, dimethyl sulfoxide (DMSO), 10% natrium chloride (NaCl), and 10% neutraled buffered formaldehyde (NBF) were obtained from the laboratory of animal experiments at the BioScience Institute, Brawijaya University (Malang, Indonesia). Alendronat sodium trihydrate as a positive control was purchased from PT. Novell Pharmaceutical Laboratories (Jakarta, Indonesia). Excipients for tablets were obtained from PT. Agaricus Sido Makmur Sentosa (Malang, Indonesia). Paraffin, xylol, alcohol, and hematoxylin and eosin were obtained from the Anatomical Pathology Laboratory, Faculty of Medicine, Brawijaya University (Malang, Indonesia).

OP Induction

A total of 35 female Wistar rats, aged 50 days, with an average body weight of 110 g, were utilized as the experimental subjects. Prior to use, the rats had a 7-day acclimatization period within the confines of the Biosciences Laboratory at Brawijaya University. The rats were provided with unrestricted access to food and water, and regular assessments were conducted to evaluate their overall health and body weight. The steps for ovariectomy are as follows [15]:

(1) The ovariectomy operation was conducted by weighing the rats to be ovariectomized and thereafter recording their weight.

(2) Following anesthesia, the rats were positioned in the left lateral position, and the skin on both the left and right sides of the rat was shaved until it was easily visible.

(3) Next, the region was cleansed with a solution of 70% ethanol. A scalpel was utilized to create an incision of 1-2 cm in length on the dorsolateral aspect of the second lumbar vertebrae, extending into the fifth or central region of the abdomen.

(4) Next, an incision measuring 1.5 to 2 cm in length was performed in the peritoneal region with either scissors or a scalpel.

(5) The left ovary and adipose tissue may be effortlessly extracted by delicately extracting them. The identical methodology is employed for the extraction of the right ovary.

CLT and alendronate treatment

The preparation of CLT suspension was carried out with grinding and the weighting of CLT obtained from PT. Agaricus Sido Makmur Sentosa, Malang, Indonesia, and prepared in suspension with doses of 24.3, 48.6, and 97.2 mg/200 g BW rats/day, whereas alendronate as a positive control was given at 0.18 mg/200 g BW rats/day. Suspension CLT and alendronate were given to the dose group orally with sonde for 2 ml

once a day during the 28 days after the rats underwent OP.

Bone specimen preparation and histomorphometric test

The surgery was carried out in several stages, starting with euthanasia, and then removing the right femur and 2–7 thoracic vertebrae, and bone fixation using a 10% NBF solution. Histological preparations were made using the hematoxylin-eosin (HE) staining method with the stages of washing, decalcification, dehydration and clearing, infiltration, embedding, slicing, staining, and mounting. A calibrated microscope was used to look at the trabecular bone preparations of a rat's femur and vertebrae to get the histomorphometry readings. When the microscope is connected to a computer, Nikon Imaging Software (NIS) elements can be used to make clear images. This can be used to measure the number of osteoblast cells by expanding them 400 times and the density of the bone mass by expanding it 100 times. Osteoblast cells are purple in colour, basophilic, have a cuboid shape, and have a mononucleus, located on the bone surface close to each other, with a shape that resembles stratified epithelial cells. Bone density was measured using ImageRaster software with a line drawing in the epiphyse line. The osteoblast cell number and bone mass density were observed and counted manually using 10 fields of view, and then the calculation results were averaged to produce the maximum amount of data.

Data analysis

Data analysis was performed by calculating the average of each treatment group, which aims to identify and compare the anti-OP activity of each CLT group compared to the negative and positive control group. The results of the research data were tested using the One-way ANOVA Test with a confidence level of 95%. Furthermore, to find out the significant differences between the tests, the LSD post-hoc test was carried out at $p < 0.05$.

Results and Discussion

The ovariectomized rat model of OP mimics the estrogen deficiency-induced bone loss and shows clinical manifestations of postmenopausal osteoporosis [15]. The ovariectomy is the removal of the ovaries, which can reduce estrogen concentrations. This is because the ovaries, as the main producer of the estrogen hormone, do not function, so estrogen levels in ovariectomy model rats will decrease drastically [16]. Estrogen deficiency also increases lifespan osteoclasts and reduces the lifespan of osteoblasts, so that the final unit balance is negative and the bone mass formed is less and results in osteoporosis. Ovariectomy further interferes with calcium absorption in the intestine and increases calcium excretion through urine. A lack of calcium in the blood causes increased resorption of calcium from the bones, leading to a decrease in bone density [15]. Estrogen deficiency after ovarectomy may increase the risk of osteoporotic kyphosis. Kyphosis is a condition where the spine curves forward. Rats whose bones are visible stooping or kyphosis is a sign of OP [17].

Rats in an OP state are characterized by the formation of kyphosis in their spine, causing the bones to become more bent, and the rats appear hunched. In addition, the signs of rats that have experienced osteoporosis include a duller appearance of fur compared to their normal state, and they may appear hunched while walking. These conditions serve as indicators of decreased bone mass.

Based on Figure 1, the bones were prepared using the HE staining method. This colouring method uses acid and base staining, which are based on the Romanowsky principle. Eosin is acidic whereas hematoxylin is basic, and acidic cell structures like the nucleus of the cell bind the colouring of the haematoxylin so that it turns blue or purple, whereas the cell structure of the base, such as the extracellular matrix and cytoplasm, binds the eosin colouring to red or pink [18].

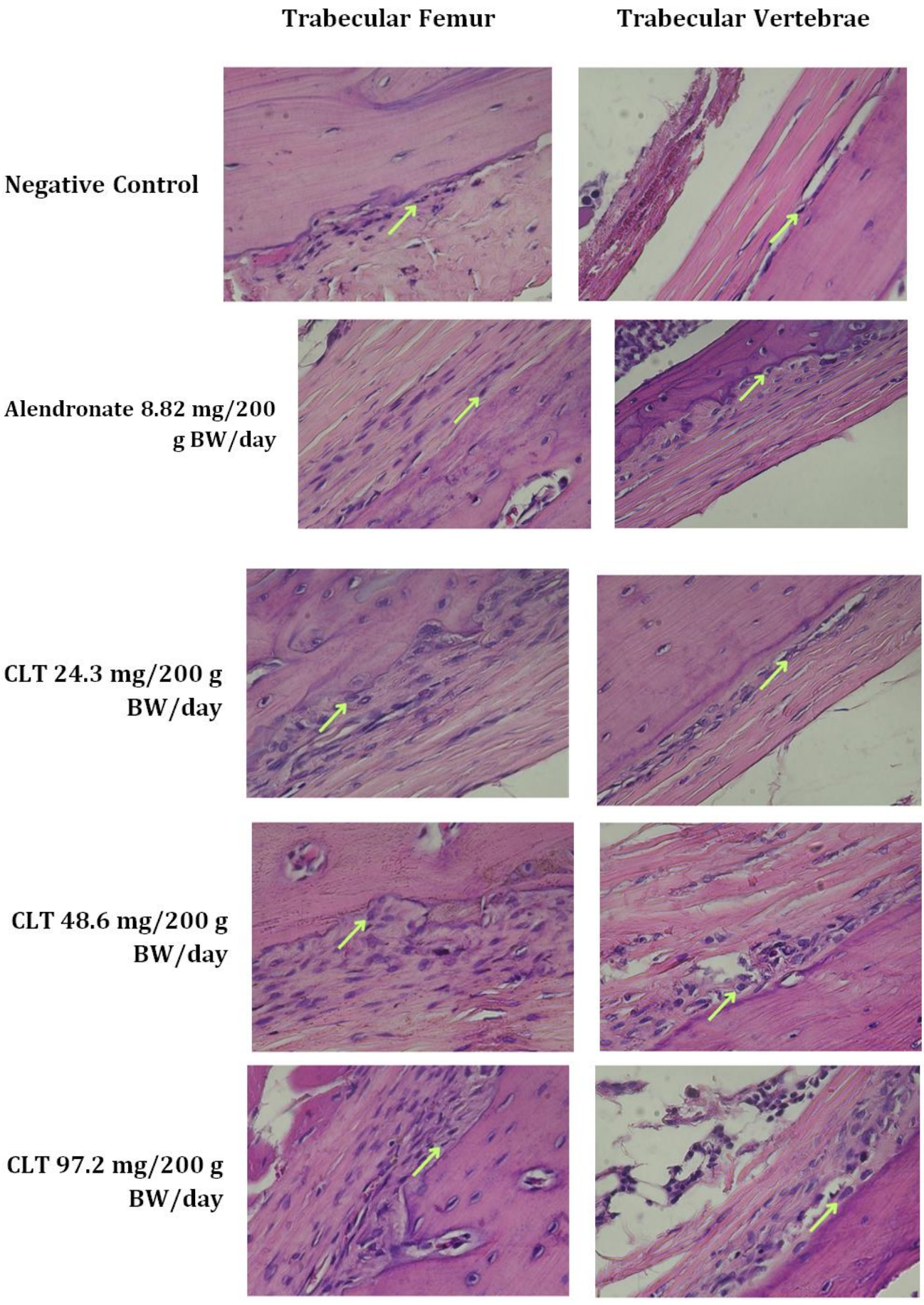


Figure 1: Observations on the morphology and osteoblast cell number (yellow arrow) in femur and vertebrae trabecular bone using a light microscope at 400x magnification

Table 1: Number of osteoblast cells in femur rats in each CLT group

Treatment groups	Osteoblast cell number
Negative control	31.58 ± 2.37
Alendronate 8.82 mg/200 g BW/day	72 ± 3.18
CLT 24.3 mg/200 g BW/day	63.54 ± 4.63a,b
CLT 48.6 mg/200 g BW/day	101.15 ± 4.12a,b
CLT 97.2 mg/200 g BW/day	73.5 ± 1.29a

Values are expressed as mean ± SD (a: significantly different from the negative control, b: significantly different from the alendronate)

Table 2: Number of osteoblast cells in vertebrae rats in each CLT group

Treatment groups	Number of osteoblast cell
Negative control	14.83 ± 2.98
Alendronate 8.82 mg/200 g BW/day	50.37 ± 8.9
CLT 24.3 mg/200 g BW/day	34.17 ± 6.92a,b
CLT 48.6 mg/200 g BW/day	71.04 ± 5.41a,b
CLT 97.2 mg/200 g BW/day	52.62 ± 6.53a

Values are expressed as mean ± SD (a: significantly different from the negative control, b: significantly different from the alendronate)

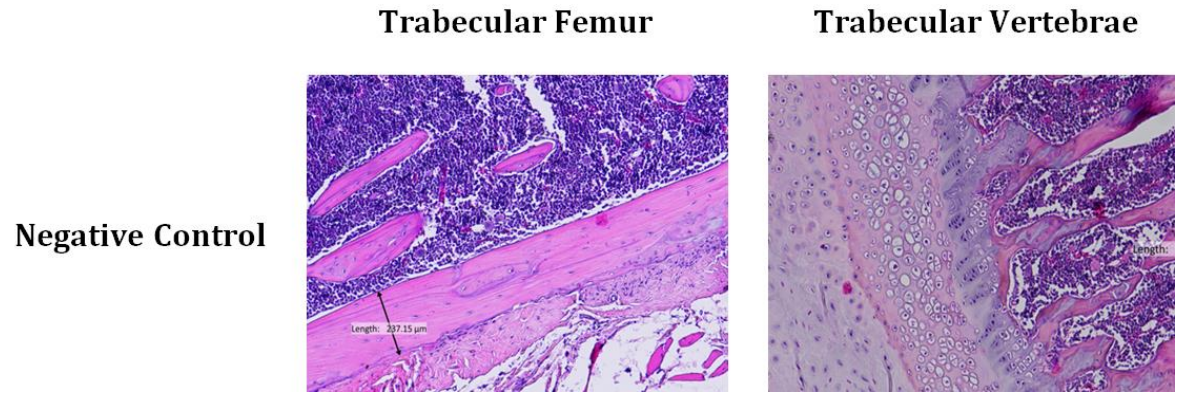
In this figure, hematoxylin gives a purple colour to the osteoblast, osteoclast, and osteocyte cells, while eosin gives a pink colour to the bone matrix. Osteoblast cells are basophilic and mononucleus cytoplasms; osteoclast cells are giant multiple cytoplasms; and osteocytes are cells that are inside the bone matrix [19].

Tables 1 and 2 indicate significant differences (p-value <0.05) in the mean values of the osteoblast cell number of the femur and vertebrae trabecular bone between negative controls with alendronate (p = 0.00), CLT 24.3 mg/200 g BW rats/day (p = 0.00), CLT 48.6 mg/200 g BW rats/day (P = 0.00), and CLT 97.2 mg/200 g BW rats/day (p = 0.00). There was also a significant difference in the osteoblast cell number of femur and vertebrae trabecular bone between alendronate with CLT 24.3 mg/200 g BW rats/day (p = 0.01) and CLT 48.6 mg/200 g BW rats/day (P = 0.00).

Based on Figure 2, measurement of bone density in trabecular bone located at the metaphysis is important because it is an active part of bone growth and affects the formation of compact bone structures and bone cavities [11].

Tables 3 and 4 show significant differences (p-value <0.05) in the mean values of the bone mass density of the femur and vertebrae trabecular bone between negative controls with alendronate (p = 0.00), CLT 24.3 mg/200 g BW (p = 0.00), CLT 48.6 mg/200 g BW (P = 0.00), and CLT 97.2 mg/200 g BW (p = 0.00).

CLT Activity on Bone Mass Density in the Femur and Vertebrae Trabecular Bone of the OP-induced Rats



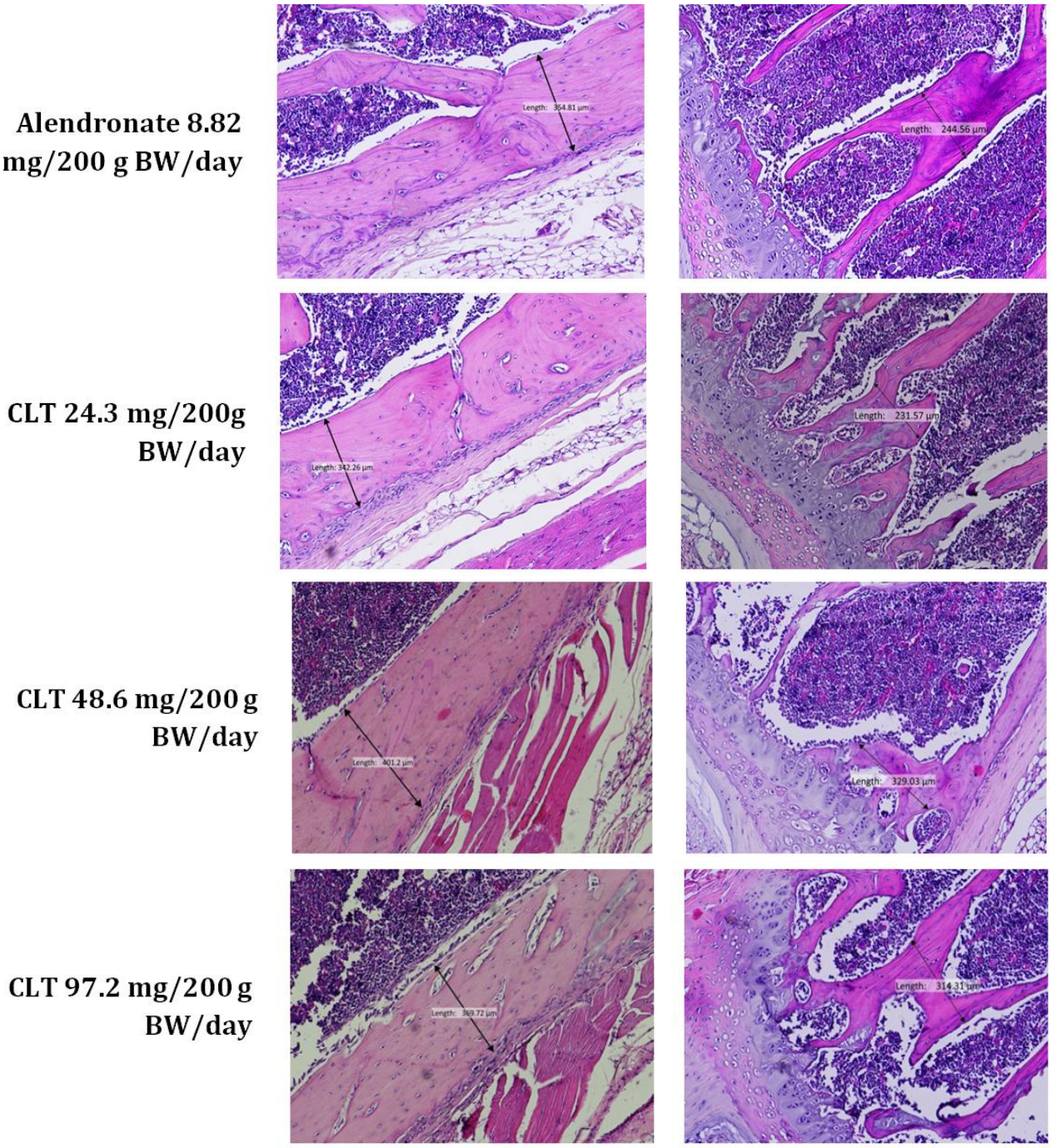


Figure 2: Observation of bone mass density (black arrow) in femur and vertebrae trabecular bone using a light microscope at 100x magnification

Table 3: Average vertebrae trabecular bone mass density of rats in each CLT group

Treatment groups	Average bone mass density value
Negative Control	97.01 ±14.54
Alendronate 8.82 mg/200g BW	214.41±29.67
CLT 24.3 mg/200g BW	211.93±21.56[a]
CLT 48.6 mg/200g BW	294.76±15.38[a,b]
CLT 97.2 mg/200g BW	274.46±35.55[a,b]

Values are expressed as mean ± SD (a: significantly different from the negative control, b: significantly different from the alendronate)

Table 4: Average femur trabecular bone mass density of rats in each CLT group

Treatment groups	Average bone mass density
Negative Control	232.53±15.15
Alendronate 8.82 mg/200 g BW	337.05±14.24
CLT 24.3 mg/200 g BW	326.09±16.65a
CLT 48.6 mg/200 g BW	428.91±10.11a,b
CLT 97.2 mg/200 g BW	412.58±17.39a,b

Values are expressed as mean ± SD (a: significantly different from the negative control, b: significantly different from the alendronate)

There was a significant difference in the mean values of femur and vertebrae trabecular bone mass density (p-value <0.05) between alendronate with CLT 24.3 mg/200 g BW (p = 0.01) and CLT 48.6 mg/200 g BW (P = 0.00). Based on the data, the best anti-OP activity is CLT 48.6 mg/200 g BW rats/day. The results of this study showed that increased doses of CLT did not result in an increase in osteoblast cell number or bone mass density, commonly called non-monotonic dose responses (NMDRs). In some cases, the biological response may undergo complex and nonlinear changes as the dose changes. It can be mentioned that a low dose of a substance can have a different effect than a high dose, or that a medium dose has a maximum effect [20]. NMDR can arise from various molecular mechanisms, such as opposite effects caused by several receptors with different affinities, receptor desensitization, negative feedback with increased doses, or dose-dependent metabolic modulation. The effectiveness of the drug will increase proportionately with the level of exposure until it reaches the upper limit, or maximum level of effect (E_{max}). An increase in doses beyond the limit will result in an increase in the dose of the poison, which leads to a decrease in effectiveness [21]. The ovariectomy process can reduce estrogen hormone levels; this is because the ovaries as the main producer of the estrogen hormone do not function, so estrogen levels in ovariectomy model rats will decrease drastically. Estrogen deficiency can increase the production of various cytokines by bone marrow stroma cells and mononuclear cells, such as IL-1, IL-6, and TNF-α, which contribute to improved osteoclastic work. The estrogen deficiency also reduces the secretion of the transforming growth factor (TGF-α), which acts as a mediator in attracting osteoblast cells to the area of the bone hole that has been absorbed by the osteoclast cell [16]. There is also an increase in RANK-L binding to the receptor, which is also followed by a decrease in the production of osteoprotegerin (OPG), resulting in an imbalance of bone remodeling processes. If this condition continues, then OP will occur and increase the risk of fractures [22].

The pharmacological effects of *C. cainito* leaves are suspected to be estrogenic properties of phytoestrogens capable of replacing estrogen for direct binding by estrogen receptors. This is because phytoestrogens have two hydroxyl groups (OH) that are between 11.0 and 11.5 A°. The distance 11 A° and the group OH are the basic structures of a substrate to have an estrogenic effect [23].

Phytoestrogen can bind to either ER-dependent or ER-independent that have different pathways to produce pharmacological effects [24]. ER-dependent has a mechanism of estrogen binding to the ER present in the cell to inhibit or activate the estrogen signal. ER-independent works in a way that hormone estrogen affects cellular signals through pathways that do not involve ER. It can bind with other receptors or proteins and activate a variety of signal paths that affect DNA transcription factors in the osteoblast or osteoclast cell [25]. Phytoestrogen activity in bone remodelling increases bone formation by osteoblast cells and reduces bone re-suppression [26].

Conclusion

CLT has anti-OP activity due to its phytoestrogen compounds, so it has the potential to be used as an antiosteoporosis alternative therapy. The chemicals in *C. cainito* leaves are thought to have

estrogenic properties because they contain phytoestrogens that can bind directly to estrogen receptors and replace estrogen in maintain bone homeostasis.

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Conflicts of Interest

The authors declare that there is no conflict of interest in this study.

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