Effects of *Eleutherine bulbosa* (mill.) urb. bulb extract on mice glucocorticoid-induced osteoporosis models

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Ethical approval and consent to participate: This research was conducted using experimental animals, which were female white rats (Rattus norvegicus) Wistar strain, obtained from the Animal Laboratory, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia. Research Ethics Commission (Animal Care and Use Committee) Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia, they have carefully studied the proposed research design, rats with healthy conditions aged 3-4 months weighing 200-300 g. Food and water were available ad libitum. Acclimatized for 1 week. Placed in a room with a 12-hour light/dark cycle with controlled conditions of temperature and humidity in the Animal Laboratory, Faculty of Pharmacy, Universitas Airlangga. Then, they hereby declare that ethically appropriate (No: 2.KEH.120.09.2022).

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Abstract

Background: Low bone mass accompanied by microarchitectural alterations in the bone that cause fragility fractures is known as secondary osteoporosis and occurs when there is an underlying condition or medication present. Eleutherine bulbosa bulb extract has been shown to affect bone because of its content, which can help osteoblast differentiation and inhibit osteoclast differentiation.

Objective: This study aimed to assess the effects of 70% ethanol extract of *E. bulbosa* Bulbs (EBE) from Pasuruan-East Java on blood calcium levels, osteoblast cell count, and bone density of trabecular femur in osteoporosis rats.

Methods: Six groups of 30 female Wistar rats were created. There were no test materials offered to the healthy group; the negative group received 0.5% CMC; the positive group received alendronate 0.9 mg/kg BW; and the dose group received 30, 60, and 120 mg/kg BW. Glucocorticoid (Dexamethasone) 0.1015 mg/kg BW/day induction was given to all groups except the healthy group to create osteoporosis rats for approximately four weeks. Then they were given oral therapy for approximately 28 days. Followed by the determination of blood calcium levels, the number of osteoblast cells, and bone density of the rat femur trabecular.

Results: The result showed that *E. bulbosa* bulbs extract could raise blood calcium levels and bone density percentage at doses of 60 and 120 mg/kg BW, as well as raise osteoblast cell levels at doses of 120 mg/kg BW.

Conclusions: The findings indicate that *E.bulbosa* bulb extract is a potential complementary medicine for osteoporosis.

Introduction

A bone metabolic disorder called osteoporosis causes diminished bone mass, alteration of bone's microarchitecture, and enhanced bone fragility, all of which raise the chance of fracture.[1] Noteworthy is that about 300,000 hip fracture patients each year wind up in nursing homes, and half never restore their pre-injury function.[2] A variety of factors can impair bone metabolism, including a lack of nutritional deficiency and sedentary lifestyle,[3] use of alcohol,[4] smoking,[5] genetic factors,[6] medication,[7] hyperparathyroidisms,[8] rheumatoid arthritis,[9] diabetes mellitus,[10] dementia,[11] and cancer.[6,12]

The glucocorticoid group is a drug in the first order that causes secondary osteoporosis, which affects adults more frequently than any other cause due to its side.[13,14] Adults on glucocorticoid often have a hunchback, back pain, height loss, or even fractures that

may result in disability, creating a significant financial burden on families and society.[14] This is because glucocorticoids affect bone mineral homeostasis with the mechanism of action of vitamin D antagonists, stimulating renal calcium excretion, and inhibiting bone formation which causes an increase in osteoclast resorption resulting in a decrease in bone mass.[15]

Corticosteroids induce osteoporosis up to eight times greater than osteoporosis due to underlying disease.[16] Induction of dexamethasone for four weeks in mice is equivalent to induction for 3-4 years for humans.[17,18] Long-term (at least 3-6 months) use of the group compounds corticosteroids may slow the process of bone growth.[19]

The *E. bulbosa* bulbs are one of the plants that contain compounds with antiosteoporosis activity. It's from the Indonesian province of Central Kalimantan.[20] Traditional use as a treatment for sprained feet.[21] This plant is from the Iridaceae family and is used to treat breast cancer and inflammatory diseases, including rheumatoid arthritis.[22,23] An in silico study published in 2014 found that *E. bulbosa* bulbs contain derivatives of the naphthoquinone compound, eleutherinol, which acts as an antagonism for mammary estrogen alpha receptors (ER-α).[20] These substances may be employed as a treatment option for postmenopausal conditions since they are anticipated to be selective agonists of estrogen receptors in different tissues, including bone and blood vessels. This extract also contains a liquiritigenin compound, which has a high affinity for selectively binding with estrogen beta receptors and can promote osteoblast differentiation while inhibiting osteoclast differentiation.[24]

As a consequence, more research is needed to scientifically prove the effects of *E. bulbosa* bulbs on osteoporosis treatment as seen by raising levels of serum calcium, the percentage of bone density, and the level of osteoblast cells.

Materials and Methods

Plant materials

Eleutherine bulbosa (Mill.) Urb. obtained from Pasuruan, East Java, Indonesia. Determined by UPT Laboratorium Herbal Materia Medica Batu, East Java, Indonesia (Certificate of Determination No. 074/722/102.7-A/2021).

Preparation of extract

The *E.bulbosa* bulbs are air-dried before being crushed into powder. The dry powder was extracted using a maceration method with 70% ethanol. A rotary evaporator was used to concentrate the extract. Ethanol extract of *E. bulbosa* bulbs was calculated as % w/w yield, which was 15.98%.

Ethical considerations

This research was conducted using experimental animals which were female white rats (*Rattus norvegicus*) Wistar strain, obtained from the Animal Laboratory, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia. Research Ethics Commission (Animal Care and Use Committee) Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia, have carefully studied the proposed research design, rats with healthy conditions aged 3-4 months weighing 200-300 g. Food and water were available *ad libitum*. Acclimatized for 1 week. Placed in a room with a 12-hour light/dark cycle with controlled conditions of temperature and humidity in the Animal Laboratory, Faculty of Pharmacy, Universitas Airlangga. Then, they hereby declare that ethically appropriate (No: 2.KEH.120.09.2022).

Animal model

Thirty rats were divided into six different groups (5 rats per group). The group was divided into[1] a healthy group, rats were not induced by glucocorticoids,[2] a negative group, rats were induced with glucocorticoids, dexamethasone (Interbat, Indonesia) 0.1015 mg/kg BW/day and given 0.5% CMC-Na therapy,[3] a positive group, rats were induced with glucocorticoids and given alendronate 0.9 mg /kg BW/day,[4] dose 1, rats were induced with glucocorticoids and given 30 mg/kg BW of EBE,[5] dose 2, rats were induced with glucocorticoids and given 60 mg/kg BW,[6] dose 3, rats were induced with glucocorticoids and given 120 mg/kg BW of extract. Glucocorticoid induction was carried out for 4 weeks orally. After the animal developed osteoporosis (kyphosis condition), therapy was carried out for 4 weeks orally. Measurements of serum calcium levels, femoral trabecular bone density, and the number of osteoblasts were performed at week 4 after therapy.

Evaluation of parameters

The level of serum calcium

At the end of the treatment, the rats in all groups were sacrificed and blood samples were taken from the heart to determine the calcium serum level. Examination of blood calcium using a spectrophotometer (λ=570 nm) at the Balai Besar Laboratorium Kesehatan (BBLK), Surabaya, Indonesia.

Histological analysis

When the procedure is over, the rats in all groups were sacrificed and the trabecular femur bone was taken. The femoral trabecular bone was prepared by fixing them with 10% formalin, decalcifying them, neutralizing them, washing them with water, and then rinsing them with 70% alcohol. After that, the bone was sealed with paraffin before being microtome-cut. Afterward, it was soaked in 70% alcohol and stained with Mallory Azan (MA). Olympus Cellsens software with a 200× zoom was used to analyze the observation slides. The microscope used for observations was connected to a computer and *Matic image software*. Bone density and osteoblast cell were calculated at the Histology Laboratory, Faculty of Medicine, Airlangga University, Surabaya, Indonesia.

Statistical analysis

SPSS was used to examine the data from the animal experiments. A Kruskal-Wallis test and a Mann-Whitney test were carried out to investigate the relationship between the treatment groups, with p-values of less than 0.05 considered to be significantly different.

Results

The level of serum calcium

The positive control group, EBE 60 mg/kg BW, EBE 120 mg/kg BW, healthy group, and negative control group all had average calcium levels that ranged from highest to lowest (Table 1).

There was a difference between the dose 1, 2, and 3 groups, as seen by the average calcium levels with dose variation ($P<0.05$). A substantial difference between EBE 30 and 60 mg/kg BW, as well as EBE 30 and 120 mg/kg BW, was revealed by statistical analysis. There was no significant difference between EBE 60 and 120 mg/kg BW.

Average values for the positive and negative control group were vastly different. According to statistical analyses, there is a significant difference between them. This is demonstrated by sta-

tistical tests that show a significant difference between the positive and negative groups. Moreover, statistical analysis revealed no significant difference in calcium levels between the positive control group and EBE (60mg/kg BW and 120 mg/kg BW). The calcium levels of the positive control group and EBE 30 mg/kg BW. High levels of calcium in the serum of the group receiving three doses of extract therapy demonstrated that the rise in the extract dose had an impact on the rise in calcium levels in the serum.[25]

Percentage of bone density

The positive control group, EBE 120 mg/kg BW, the healthy group, EBE 60 mg/kg BW, EBE 30 mg/kg BW, and the negative control group had an average bone density from highest to lowest (Table 1). The healthy group's average bone density is significantly higher than that of the negative control group. Statistical tests that demonstrate a substantial difference in bone density between the healthy group and the negative control group serve as proof of this. This shows that the glucocorticoid induction process was successful and that rats were experiencing osteoporosis. The value for a healthy group is 50.65±6.42 and for a negative control is 37.70±7.54 (mean ± SD). In comparison to the negative control group, the positive control group had an average bone density that was considerably higher. This is supported by statistical analyses that show a significant difference between the positive and negative control group. The level of bone density between of positive control group and EBE (60 and 120 mg/kg BW) did not differ significantly, according to statistical analysis. Additionally, there was a statistically significant difference in bone density between the EBE 30 mg/kg BW group and the positive control group.

Level of osteoblast cell

The positive control group, EBE 120 mg/kg BW, EBE 60 mg/kg BW, the healthy group, EBE 30 mg/kg BW, and the negative control group had an average level of osteoblast cells from highest to lowest (Table 1). There was a difference in the number of osteoblast cells with the various dose changes in the extract dose treatment group. The statistical test results revealed that EBE 60 and 120 mg/kg BW did not differ significantly from each other, while EBE 30 and 120 mg/kg BW; EBE 30 and 60 mg/kg BW did differ significantly from each other.

The average level of osteoblast cells was considerably greater in the positive control group than in the negative control group. Statistical tests demonstrating a significant difference between the positive control group and the negative control group serve as proof of this. Additionally, statistical analysis revealed no significant difference between EBE 120 mg/kg BW and the positive control group's osteoblast cell levels. Moreover, there were significant differences between the osteoblast cell levels of the positive control group and EBE (30 mg/kg BW, 60 mg/kg BW).

Discussion

Direct inhibition of osteoblast proliferation, hyperparathyroidism brought on by direct effects on the parathyroid gland, a rise in urinary calcium excretion linked to glucocorticoids, and direct stimulation or inhibition of osteoclast formation are some of the multiple ways that glucocorticoids affect bone metabolism.[26,27] Similar to glucocorticoid-induced osteoporosis, this condition is characterized by a raise in the angle of the spine or is called a state of kyphosis in animal models. Osteoporosis can also be proven in the trabecular femur by looking at the parameters of decreasing bone volume density (BV/TV) and bone mineral density (BMD).[28]

The percentage of bone density and osteoblast cell levels differed significantly between the healthy and negative groups (P<0.05), it could be seen that the glucocorticoid can decrease the percentage of bone density and osteoblast cell levels. The dose treatment group then demonstrated the opposite effect, increasing the percentage of bone density and osteoblast cell levels.

The chemicals included *E. bulbosa* bulbs enabled it to raise both the percentage of bone density and osteoblast cell level. *E. bulbosa* bulbs are reported to contain 2,4,7-Trihydroxy-9,10-dihydrophenanthrene (phenanthrene), cuspidatumin A (naphthoquinone), dendromoniliside E (glycoside), liquiritigenin (flavonoid), and natsudaidain (flavonoid).[29] In vitro studies have shown that liquiritigenin raises osteoblast activity and reduces osteoclast differentiation.[30] Furthermore, fish scales' natural bone metabolism can be preserved by liquiritigenin.[31] Liquiritigenin was found to be able to stimulate dose-dependent osteoblast development by working on the Smad1/5 pathway, boosting ALP activity, collagen synthesis, and mineralization in a study utilizing MC3T3-E1 cells.[32,24]

The average calcium level in the healthy group found no significant differences between the negative group and EBE 30 mg/kg BW (P>0.05). However, there were significant differences between the positive control, EBE 60 and 120 mg/kg BW (P<0.05). The results of this study prove that EBE 60 and 120 mg/kg BW can increase serum calcium levels when compared to the healthy group. Increased calcium content in serum and plasma is a sign of a variety of diseases, one of which is primary hyperparathyroidism (pHPT). Secondary osteoporosis is caused by pHPT, which results in low bone mineral density (BMD).[33] BMD is primarily used to diagnose ostoeporosis.[34]

Limitations

The study's limitations were recognized. No examination was performed when glucocorticoid induction was completed. However, from the previous reference, it was stated that it took about four weeks to obtain a state of osteoporosis after induction with a change in posture in the spine to a hunchback (kyphosis) (based on preliminary research).

Table 1. Analysis result of blood and bones. Mann-Whitney test was used for statistical comparison between treatment and healthy groups (n=5).

Treatments	Average ± SD					
	Healthy group	Negative control (0.5% CMC-Na)	Positive control (Alendronat 0.9 mg/kg BW)	EBE (30 mg/kg BW)	EBE (60 mg/kg BW)	EBE (120mg/kg BW)
Serum calcium level	10.0±0.39	9.9±0.13	11.0±0.23*	9.9±0.17	10.8±0.18*	10.7±0.23*
Bone density Percentage	50.65±6.42	37.70±7.54*	65.48±7.61*	39.69±1.31*	48.43±14.49	60.67±10.54
Osteoblast cells level	125.8±16.99	49±25.00*	192.8±3.27*	96.4±5.31*	130.6±15.70	146.6±47.76

*P<0.05.

Conclusions

In summary, it is inferable that EBE bulbs have an effect on raising serum calcium levels, bone density (the best effect by EBE 60 and 120 mg/kg BW), and raising the number of osteoblast cells (EBE 120 mg/kg BW). According to these results, EBE at this dosage of 120 mg/kg BW may be just as effective at treating osteoporosis as alendronate.

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