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EFFECTS OF *BLAINVILLEA ACMELLA* ON THE BONE MECHANICAL PROPERTIES OF OVARIECTOMIZED RATS

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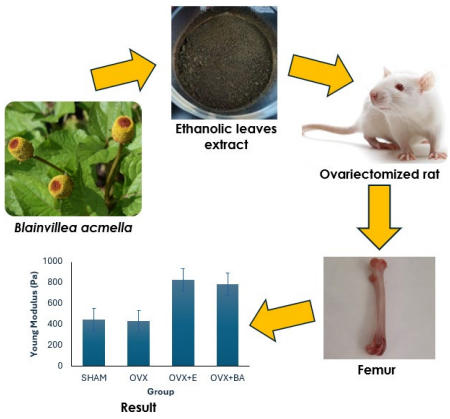
Graphical abstract



Abstract

A metabolic bone disorder known as osteoporosis is represented by low mineral bone mass and strength. *Blainvillea acmella* (BA), a herbal plant with antioxidant properties have exhibited anti-osteoporosis effects. The present study set out to discover the osteoprotective effects of BA on bone mechanical properties of the ovariectomized rat model of osteoporosis. Twenty-four Sprague-Dawley female rats were designated into four groups (n=6 per group), namely, (SHAM) sham-operated, (OVX) ovariectomized control, (OVX+E) ovariectomized and supplemented with Premarin 64.5 µg/kg and (OVX+BA) ovariectomized and supplemented with 100 mg/kg of BA ethanolic leaves extract. All rats were euthanized after 12 weeks and the right femurs were tested in three-point bending using Instron Universal Test Machine (Shimadzu AG-X 500N). The bone mechanical properties were evaluated in two parts: extrinsic factors (load, displacement and stiffness) as well as intrinsic factors (stress, strain and Young modulus). The maximum stress and Young modulus of OVX+E and OVX+BA groups indicated considerably higher levels compared to SHAM and OVX groups (p<0.05). No significant differences were found in bone strength between OVX+E and OVX+BA groups. In conclusion, BA leaves extract was comparable to Premarin in maintaining optimal bone strength of the ovariectomized rat model of osteoporosis. Therefore, BA potential in treatment of osteoporosis warrants further investigation.

Keywords: Bone mechanical properties, *Blainvillea acmella*, osteoporosis, ovariectomised rat, three-point bending

Abstrak

Gangguan metabolisme tulang yang dikenali sebagai osteoporosis dijelaskan dengan keadaan jisim dan kekuatan mineral tulang yang rendah. *Blainvillea acmella* (BA), sejenis tumbuhan herba dengan sifat antioksidan telah menunjukkan kesan anti-osteoporosis. Kajian ini bertujuan untuk menentukan kesan osteoprotektif BA terhadap sifat mekanikal tulang pada model osteoporosis tikus terovariektomi. Sebanyak dua puluh empat ekor tikus betina *Sprague-Dawley* telah ditetapkan kepada empat kumpulan (n=6 setiap kumpulan), iaitu, (SHAM) kawalan Sham, (OVX) kawalan ovariektomi, (OVX+E) terovariektomi dan ditambah dengan Premarin 64.5 µg/kg dan (OVX+BA) terovariektomi dan ditambah dengan 100 mg/kg ekstrak etanol daun BA. Semua tikus telah dibunuh selepas 12 minggu dan tulang femur kanan telah diuji dengan pelenturan tiga titik menggunakan Mesin Ujian Universal Instron (Shimadzu AG-X 500N). Sifat mekanikal tulang dinilai dalam dua bahagian: faktor ekstrinsik (beban, anjakan dan kekakuan) serta faktor intrinsik (tekanan, regangan dan Modulus kekenyalan). Tekanan maksimum dan Modulus kekenyalan bagi kumpulan OVX+E dan OVX+BA menunjukkan tahap yang jauh lebih tinggi berbanding kumpulan SHAM dan OVX (p<0.05). Tiada perbezaan ketara ditemui dalam kekuatan tulang antara kumpulan OVX+E dan OVX+BA. Kesimpulannya, ekstrak etanol daun BA adalah setanding dengan Premarin dalam mengekalkan kekuatan tulang optimum model osteoporosis tikus terovariektomi. Oleh itu, potensi BA dalam rawatan osteoporosis memerlukan kajian selanjutnya.

Kata kunci: Sifat mekanikal tulang, *Blainvillea acmella*, osteoporosis, tikus terovariektomi, pelenturan tiga titik

1.0 INTRODUCTION

The systemic skeletal disease known as osteoporosis is marked by reduced bone mineral density and deteriorating microarchitecture, leading to decreased bone mass and strength [1]. Osteoporosis is a significant public health issue in developing nations, including Malaysia. Hip fractures are prevalent and debilitating among the elderly, especially in women, and their global incidence is expected to increase. Furthermore, one in three women worldwide experiences osteoporotic fractures after the age of 50 [2]. Despite variations in epidemiological data across countries, it is widely accepted that hip fractures affect approximately 6% of men and 18% of women globally [3].

Epidemiological data on osteoporosis in Malaysia are sparse. Given that osteoporosis is the leading cause of hip fractures in the elderly, studying the epidemiology of hip fractures among ageing Malaysian population could help estimate the prevalence of osteoporosis. The population in Malaysia aged over 50 was projected to rise from 5.3 million in 2013 to 13.9 million by 2050 [4]. According to recent projections, the incidence of hip fractures in Malaysia is anticipated to rise by 3.55 times by 2050 compared to the rates in 2018 [5]. This trend is concerning as hip fractures can lead to significant societal costs and other adverse effects such as depression, disability, and cardiovascular disease.

Bone resorption and formation are the fundamental processes of bone metabolism, crucial for acquiring and maintaining bone mass, structure, and quality. Osteocytes act as mechanosensors and coordinators of bone remodeling, with osteoclasts and osteoblasts working together to continuously renew bone tissue. These cells regulate blood calcium levels and overall bone health. Low bone mineral density can result from excessive osteoclast activity or insufficient osteoblast activity, potentially leading to osteoporosis. An inequality between bone formation and resorption, along with increased bone turnover, can cause rapid bone loss [6].

Estrogen is a key hormonal regulator of bone metabolism in women [7], playing a significant role in determining bone mass. In order to regulate bone remodeling, estrogen interacts with the estrogen receptors in bone cells. The majority of postmenopausal women experience bone loss, which have often been linked to the lower levels of estrogen [6]. Hormone replacement therapy (HRT), such as Premarin, can reverse estrogen deficiency and protect postmenopausal women against bone loss. Treatment with estradiol, a form of estrogen, has improved cortical and trabecular thickness as well as bone strength of the rats [8]. Nevertheless, continuing HRT use is correlated with an increase risk of developing cancers [9]. Bisphosphonates, synthetic drugs used to reduce fracture risk, can cause jaw osteonecrosis [10]. Selective estrogen receptor modulators (SERMs) may lead to thromboembolic disorders and other side effects [11, 12].

To avoid the side effects of synthetic drugs, alternative therapies for osteoporosis have been explored, including natural remedies like Labisia pumila var. alata [13], virgin coconut oil [14], and *Apis*

dorsata [15]. *Blainvillea acmella* (L.) Philipson, a plant native to the genus Spilanthes and Asteraceae family [16], is commonly known in Malaysia as "Pokok Getang" or "Subang Nenek." It is native to various regions including Andaman Island, Cambodia, and China South-Central. *Blainvillea acmella* has a potential anti-malaria property [17] and is used in dietary supplements and cosmetics [18]. Its efficacy in treating blood disorders is attributed to its antibacterial, antioxidant, and vasorelaxant properties [19].

Several studies have reported the bone anabolic activities of *Blainvillea acmella*. Widyowati *et al*. (2011) demonstrated that *Blainvillea acmella* leaves extract increased alkaline phosphatase levels, a marker for bone formation [20]. Later, its ability to promote bone mineralization was confirmed [21]. A recent in vitro study revealed *Blainvillea acmella*'s bone-anabolic activities, including bone proliferation, differentiation, and mineralization [16]. These activities were positively correlated with the plant's phenolic content and antioxidant properties. Phytochemical analyses identified compounds like terpenoids and flavonoids in *Blainvillea acmella* contributing to its effects. The bone anabolic activities of *Blainvillea acmella* could offer protection against osteoporotic fractures by strengthening load-bearing bones like the spine and hip. Bone strength can be measured using biomechanical testing, which requires animal models. The three-point bending test is commonly used due to its accessibility.

No extensive studies have been conducted on the effects of *Blainvillea acmella* on bone in animal models. The major purpose of study is to find out the effects of *Blainvillea acmella* ethanolic leaves extract on bone mechanical properties in the ovariectomized rat model of osteoporosis, using three-point bending test. This will evaluate its potential as an anti-osteoporotic agent.

2.0 METHODOLOGY

2.1 Animal and Treatment

The study was carried out between April 2022 and July 2023 at Universiti Teknologi MARA, Sungai Buloh Campus, Selangor, Malaysia. Twenty-four female Sprague-Dawley rats (250-300g) were bought from A Sapphire Sdn. Bhd., Seri Kembangan, Selangor, Malaysia. The rats were allocated into four groups of six at random. All rats were fed a rat chow diet and given reverse osmosis water ad libitum. Ovariectomy was performed to induce an estrogen-deficient state, mimicking postmenopausal osteoporosis, as described by Yousefzadeh *et al*. [22]. The sham-operated (SHAM) and ovariectomized-control (OVX) groups received normal saline water as a vehicle. The treated groups received 64.5 µg/kg of Premarin (OVX+E) [23] and 100 mg/kg of *Blainvillea acmella* ethanolic leaves extract (OVX+BA) according to the preliminary study by Laswati *et al*. [24]. All rats were euthanized 12 weeks later, and the right femurs were collected for further analysis. The success of ovariectomy was confirmed by the atrophied uterine horns that were observed in the ovariectomized rats during necropsy. Ethical approval was granted by the Committee on Animal Research and Ethics (CARE), UiTM (Ethic approval number: UiTM CARE 383/2022).

2.2 Specimen Collection

After receiving treatment for 12 weeks, all rats were anesthetized with high doses of ketamine/xylazil combination. After being dissected, all soft tissues were removed from the right femurs. Each femur was enveloped in PBS-soaked gauze and aluminum sheet, then stored frozen at -80°C until analysis. Before testing, all femurs were allowed to thaw for two hours at room temperature (28°C).

2.3 Bone Biomechanical Test

The bone mechanical properties of the right femurs were evaluated using a three-point bending setup on the Instron Universal Test machine (Shimadzu, AG-X 500N Series) with Trapezium X software (Figure 1). The full-length and midshaft wideness of the femur were calibrated with a digital vernier caliper. The femur was positioned on two lower jig support points, spaced 10 mm apart (Figure 2). Mechanical loading was applied to the femoral mid-diaphysis at a pace of 10 mm/sec until the femoral shaft fracture. Extrinsic (load, displacement) and intrinsic (stress, strain) parameters were documented. The geometry and mechanical properties of the bone were derived, including stiffness (the slope of load-displacement curve) together with Young modulus (the slope of stress-strain curve).

2.4 Statistical Analysis

The SPSS 27.0 Software was used to analyze the data. Normality was evaluated with the Kolmogorov-Smirnov test. One-way ANOVA followed by Tukey's HSD test were used for normally distributed data. Both Kruskal-Wallis and Mann-Whitney tests were used to analyze the data that did not follow a normal distribution. Results were expressed as mean ± SEM. $p<0.05$ was indicated statistically significant differences.

Figure 1 Instron Universal Test Machine (Shimadzu AG-X 500N Series)

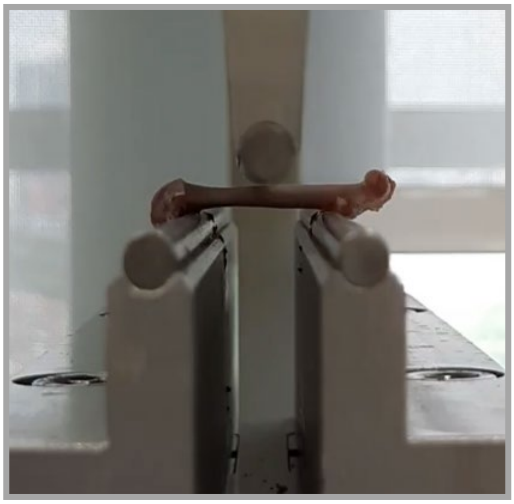


Figure 2 Three-point bending, in which the femur was positioned on two lower jig support points

3.0 RESULTS AND DISCUSSION

Bone tissue is composed of four types of cells: osteocytes, osteoblasts, osteoclasts, and osteoprogenitor cells. Bone undergoes continuous remodeling through osteoblasts' bone formation and osteoclasts' bone resorption. Osteocytes, the most abundant bone cells, act as mechanotransducers in this process. Osteoprogenitor cells are involved in bone repair and growth. Bone mass is regulated by the dynamic balance of these cells, influenced by estrogen levels. Estrogen, a natural hormone, is a key regulator of bone turnover. Its deficiency after menopause contributes to the significant loss of bone strength and increasing osteoporosis risk factors [25].

HRT has been used to supplement estrogen and prevent menopause-related bone loss in women, but it carries risks of various cancers [9]. Due to these complications, antioxidant supplementation has been explored as a potential osteoporosis therapy. In this study, Premarin (64.5 µg/kg) and *Blainvillea acmella* ethanolic leaves extract (100 mg/kg) were administered to treated groups.

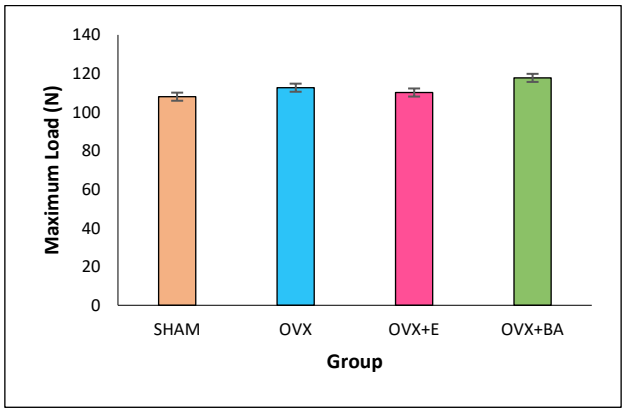
Blainvillea acmella leaves extract, known for its antioxidant properties [26], was chosen as an alternative treatment for ovariectomized rats. Previous studies showed *Blainvillea acmella*'s effective antioxidant activity [27], attributed to bioactive compounds like phenolics, coumarins, and triterpenoids [28]. This study aimed to evaluate *Blainvillea acmella*'s impact on bone strength and its anti-osteoporotic effects in a rat model. Biomechanical testing using three-point bending analyzed bone mechanical properties and strength.

Biomechanical testing measures bone strength, crucial for assessing fracture risk in osteoporosis. Various approaches like compression, tension, torsional, or bending can be used, with bending often preferred for smaller bones like the femur. Extrinsic factors (load, displacement, stiffness) reflect external influences on the entire bone, while intrinsic factors (stress, strain, Young modulus) relate to internal factors like bone geometry and metabolism.

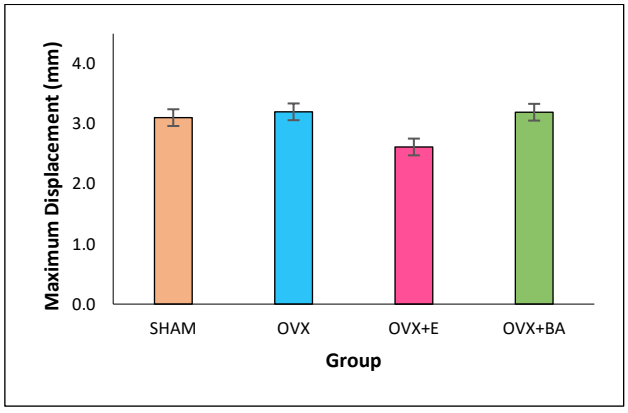
Extrinsic factors showed no significant differences between groups (Figure 3), aligning with previous studies using combined natural products [29]. Maximum displacement, indicating femur movement under external force, also showed no significant differences. The lack of significant changes in parameters like maximum displacement and strain between OVX and SHAM groups, despite expected reductions post-ovariectomy, has been noted in other studies [30]. Variations in testing machines, rat strains, and treatment durations could contribute to inconsistent findings. The mid-diaphyseal femur region's cortical bone changes less dramatically than trabecular bone, possibly requiring longer post-ovariectomy periods for consistent results. The results of extrinsic factors are shown in Table 1.

Table 1 Summary, bone mechanical properties: means and standard deviations of extrinsic factors; maximum load (N), maximum displacement (mm) and maximum stiffness (N/mm) of the femoral mid-diaphysis

	Extrinsic factors		
	Maximum load (N)	**Maximum displacement (mm)**	**Maximum stiffness (N/mm)**
SHAM (n=6)	108.00 ± 10.22	3.10 ± 0.48	35.30 ± 4.91
OVX (n=6)	112.65 ± 1.68	3.20 ± 0.95	38.04 ± 12.30
OVX+E (n=6)	110.16 ± 6.35	2.62 ± 0.75	43.81 ± 7.56
OVX+BA (n=6)	117.74 ± 10.40	3.19 ± 0.36	37.00 ± 2.04



(a)



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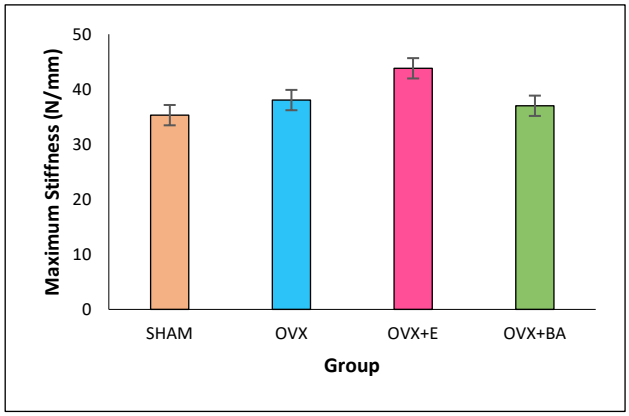


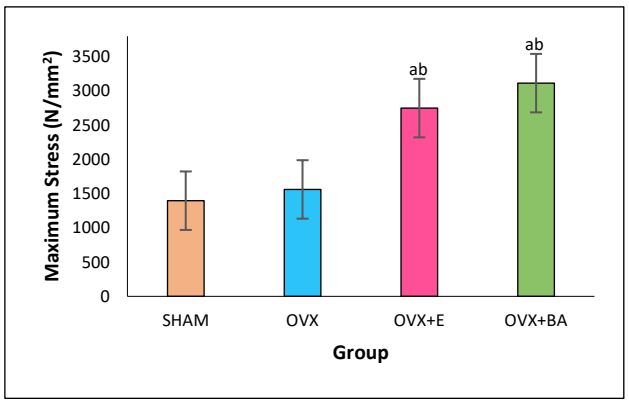
(c)

Figure 3 Extrinsic factors; (a) maximum load; N, (b) maximum displacement; mm, and (c) maximum stiffness; N/mm, of the femoral mid-diaphysis.

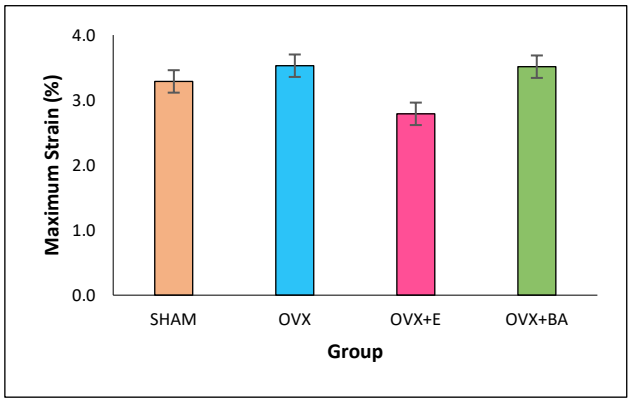
Intrinsic factors showed better mechanical properties, with significant differences in maximum stress and Young's modulus (Figure 4). Maximum stress indicates bone tissue strength under load before fracture. Both OVX+E and OVX+BA groups had significantly higher maximum stress than SHAM and OVX groups, consistent with previous studies [31]. Young's modulus, derived from the stress-strain curve, reflects bone's resistance to deformation. Higher values in treated groups suggest *Blainvillea acmella*'s effectiveness in protecting bone intrinsic properties against estrogen deficiency. The results of intrinsic factors are shown in Table 2.

Table 2 Summary, bone mechanical properties: means and standard deviations of intrinsic factors; maximum stress (N/mm^2), maximum strain (%) and Young Modulus (Pa) of the femoral mid-diaphysis.

	Intrinsic factors		
	Maximum stress (N/mm^2)	**Maximum strain (%)**	**Young Modulus (Pa)**
SHAM (n=6)	1397.20 ± 39.67	3.29 ± 0.52	446.43 ± 28.27
OVX (n=6)	1561.60 ± 45.60	3.53 ± 1.05	429.93 ± 57.78
OVX+E (n=6)	2749.87 ± 40.76	2.79 ± 0.68	826.54 ± 13.95
OVX+BA (n=6)	3114.51 ± 23.19	3.51 ± 0.55	787.34 ± 11.75



(a)



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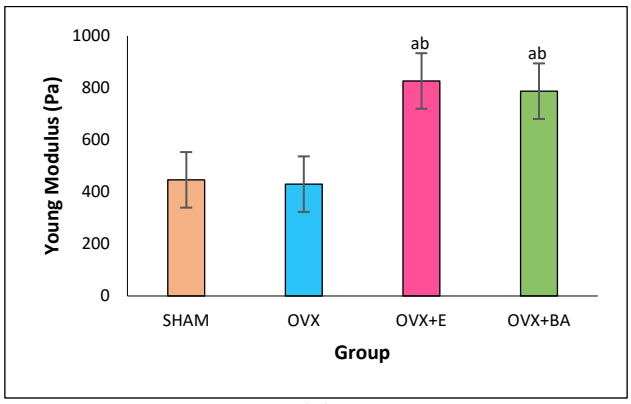

(c)

Figure 4 Intrinsic factors; (a) maximum stress; N/mm², (b) maximum strain; %, and (c) Young Modulus; Pa, of the femoral mid-diaphysis. [a]Significantly different from SHAM group (p<0.05), [b]Significantly different from OVX group (p<0.05).

Blainvillea acmella's antioxidant activity, due to bioactive compounds like phenolics, coumarins, and triterpenoids, likely contributes to its bone-anabolic effects. Recent studies confirmed *Blainvillea acmella*'s positive impact on bone proliferation, differentiation, and mineralization [32], reducing fracture risk by enhancing bone formation and mineralization.

This study found that 100 mg/kg of *Blainvillea acmella* ethanolic leaves extract improved maximum stress and Young modulus in ovariectomized rats, comparable to Premarin. *Blainvillea acmella* showed potential as an effective anti-osteoporotic agent, protecting bone strength.

4.0 CONCLUSION

Blainvillea acmella ethanolic leaves extract demonstrated a protective effect on bone strength in ovariectomized rats, suggesting its potential for osteoporosis prevention. However, further studies are needed to elucidate its mechanisms of action in treating osteoporosis in postmenopausal women.

Limitations

There was limitation in this study as a result of lack of scholarly paper addressing the therapy of *Blainvillea acmella*. The previous studies have focused more on *in vitro* only. In addition, there are also no study that focused on animal studies treated by *Blainvillea acmella* ethanolic leaves extraction. Therefore, we believed that valuable evidence concerning the protective effects of *Blainvillea acmella* ethanolic leaves extraction in osteoporosis will be helpful in treating the bone damage of the ovariectomised rats.

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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