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Original Article

Bilobetin Mitigates Osteoporosis in Ovariectomy-Induced Rats by Modulating Bone Remodeling Markers, RANKL/OPG, and NF-κB/SIRT1 Pathways

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ABSTRACT

Background: Osteoporosis is a common skeletal condition marked by decreased bone density and impaired bone microarchitecture, which substantially increases the fracture risks. This systemic condition results from a disproportion in bone homeostasis, when bone resorption exceeds bone formation. **Objectives:** This work focuses to study the anti-osteoporotic efficacy of bilobetin against Overiectomy (OVX)-induced osteoporosis in rat model. **Materials and Methods:** The OVX surgery was done on the experimental rats to develop osteoporosis, followed by a 12-week treatment with bilobetin. The biomechanical parameters were evaluated in the femur bones of experimental rats. The concentrations of bone turnover biomarkers were evaluated using kits. Furthermore, the levels of Calcium (Ca) homeostasis markers, oxidative stress indicators, inflammatory biomarkers, and bone remodeling marker proteins were evaluated using kits. **Results:** The results of this work indicated that bilobetin treatment effectively regulated the biomechanical parameters of femur bones, bone turnover markers, and calcium homeostasis in the OVX-induced rats. Furthermore, bilobetin treatment also decreased the inflammatory biomarkers, reduced oxidative stress responses via elevating antioxidants, increased Runx2 and OPG concentrations, and reduced RANKL levels in OVX rats. **Conclusion:** The present findings elucidated the anti-osteoporosis effects of bilobetin against OVX-induced rats. The present data indicated the advantageous effects of bilobetin in mitigating the osteoporosis condition in OVX rats, thereby establishing it as a promising pharmacological treatment for osteoporosis management.

Keywords: Bone Remodeling, Calcium Homeostasis, Bilobetin, Overiectomy, RANKL/OPG Pathway.

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INTRODUCTION

Osteoporosis, a skeletal disease defined by diminished Bone Mineral Density (BMD) and compromised bone microarchitecture, significantly elevates fracture risk. This systemic condition arises from an imbalance in bone homeostasis, wherein osteoclast-regulated bone resorption surpasses osteoblast-regulated bone development.[1] The consequences of osteoporosis extend beyond mere bone fragility, impacting quality of life, increasing disability, and contributing to substantial healthcare expenditures. Osteoporosis is often asymptomatic until a fracture occurs, highlighting the importance of early detection and intervention. Advanced age, genetics, lifestyle factors, and certain medical conditions participates in the onset of osteoporosis.[2] The prevalence of osteoporosis is substantial, particularly among aged people, with women disproportionately affected due to hormonal changes associated with menopause. It is estimated that a significant proportion of women and men will experience at least one osteoporotic fracture in their lifetime, underscoring the far-reaching impact of this disease. Advanced imaging modalities, including quantitative computed tomography, provide enhanced evaluations of bone microarchitecture that extend beyond mere BMD.[3] This is particularly important since BMD measurements may not fully reflect bone quality. The complex pathophysiology of osteoporosis involves intricate interactions between various cell types, signaling pathways, and hormonal influences. These interactions ultimately determine bone remodeling rates and overall bone strength.[4]

Current treatment strategies for osteoporosis encompass lifestyle modifications, pharmacological interventions, and fall prevention measures. Lifestyle modifications, such as sufficient calcium and vitamin D consumption, consistent weight-bearing activity, and cessation of smoking, are essential for preserving bone health and preventing further bone loss. Pharmacological interventions

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like antiresorptive drugs, including bisphosphonates, selective estrogen receptor modulators, and denosumab, which inhibit osteoclast activity and reduce bone turnover.[5] Anabolic agents, such as teriparatide, enhance osteoblast activity and stimulate new bone development, offering an alternative approach to managing osteoporosis. Despite the availability of effective treatments, challenges remain in optimizing therapeutic strategies and addressing individual patient needs. The rapidly expanding population over the age of 65 is likely to increase the influence of osteoporosis on healthcare. Adherence to long-term osteoporosis treatment can be challenging, with many patients discontinuing therapy due to side effects, complex dosing regimens, or perceived lack of benefit.[6] There is a critical need for more effective strategies to detect individuals at high fracture risk and ensure timely intervention. There is limited evidence for therapies targeting both resorption and formation. Further research is needed to develop novel therapeutic targets and personalized treatment approaches that address the underlying mechanisms of osteoporosis and improve long-term outcomes.[7]

The Ovariectomy (OVX)-induced osteoporosis stands as a pivotal tool in preclinical research for evaluating the efficacy of novel therapeutic interventions, particularly plant-bioactive compounds and sample drugs, aimed at combating osteoporosis. The OVX model, which mimics the hormonal changes associated with menopause in humans, provides a controlled and reproducible experimental platform to investigate the pathophysiology of osteoporosis and to assess the potential of new treatments to prevent or reverse bone loss.[8] By surgically removing the ovaries, researchers can induce a state of estrogen deficiency, resulting in accelerated bone resorption and reduced bone growth, thereby replicating the key features of postmenopausal osteoporosis. This model is valuable because it allows for the direct examination of how estrogen deficiency affects bone metabolism and how potential treatments can counteract these effects.[9] The utilization of the OVX-induced osteoporosis model in rats is essential for the preclinical screening and evaluation of plant-bioactive compounds with potential anti-osteoporotic properties. Plant-derived compounds represent a rich source of novel therapeutic agents, and many have demonstrated promising effects on bone health. By using the OVX model, researchers can assess whether these compounds can prevent bone loss, stimulate bone formation, or both, providing critical data for guiding further drug development.[10] Bilobetin is a natural biflavonoid compound extracted from some gymnosperm plants, including *Ginkgo biloba*. It has already been mentioned that bilobetin has several biological activities, including antifungal, antioxidant, antiinflammatory, anti-proliferative, and antihyperlipidemic activities.[11] Furthermore, bilobetin ameliorated insulin resistance[12] and protected against cisplatin-induced testicular toxicity[13] in rat models. Nevertheless, the anti-osteoporotic efficacy of bilobetin has not yet been scientifically proven. Consequently, this study aims to investigate the anti-osteoporotic property of bilobetin against OVX-induced osteoporosis in the rat model.

MATERIALS AND METHODS

Chemicals

The primary chemicals utilized in this study, including bilobetin and alendronate, were sourced from Sigma Aldrich (USA). The diagnostic kits utilized to measure the concentrations of biochemical markers were procured commercially from MyBioSource, ELK Biotechnology, and Elabscience, USA, respectively.

Experimental animals

The current work employed healthy female Sprague Dawley rats with an average weight of approximately 210±30 g. The rats were habituated in a regulated laboratory setting for seven days. The rats were housed in sterile polypropylene enclosures. Rigorous hygiene standards were maintained throughout the duration of the studies, with cages and bedding being routinely substituted. Standard temperature and relative humidity were consistently upheld, and a 12-hr light/dark series was implemented with free access to standard diet and water.

Establishment of OVX model

After a 12-hr fasting period, animals were sedated by injecting pentobarbital sodium (3%; 35 mg/kg). Animals were subsequently positioned onto the surgical plate. The OVX method was performed using the dorsal method, wherein the dorsal region was shaved and sanitized with ethanol (75%). The sterile incision of 1 cm was made, and the ovaries were ligated and excised. The surgical wound was closed with many layers, and penicillin and lidocaine were applied topically to the spot. The SHAM-operated animals removed comparable quantities of adipose tissue situated near the ovaries. The model was created 12 weeks after ovariectomy. The results demonstrated that the uteri of rats in the OVX group displayed atrophy and notable morphological abnormalities relative to the SHAM, signifying effective modeling as reported previously.[14]

Experimental groups

Upon successful recovery from surgery, the experimental rats were allocated into four experimental groups, each including six animals (n=6). Group I received a placebo procedure and were provided with the regular diet. Group II consists of rats that underwent the OVX procedure and were monitored for a week. Group III consists of rats with OVX induction and that received bilobetin at a dose of 10 mg/kg. Group IV had OVX and was treated with alendronate (2.5 mg/kg). The bilobetin or alendronate treatment was initiated in the fourth week post-surgery and persisted for 12 weeks. Upon completion of

the treatments, the animals were anesthetized, euthanized, and subsequently, samples were procured for further investigations.

Evaluation of biomechanical parameters

The femur bone was employed to evaluate biomechanical parameters. The material testing machine (Shimadzu, Japan) was employed to perform a three-point bending test. The caliper was employed to measure the length of the femur. The femur was positioned on the points (19 mm), maintaining a comparable configuration. A force was subsequently applied to the central portion of bone utilizing a crosshead that traversed at a 1 mm/minute velocity. The assessment of displacement and central load was performed until the occurrence of fracture. The parameters, including maximum load, stress, Young's modulus, and energy absorption, were computed utilizing a previous methodology.[15]

Evaluation of bone turnover markers

The concentrations of bone turnover biomarkers, namely Alkaline Phosphatase (ALP), bone-specific Acid Phosphatase (ACP), Beta-CrossLaps (β-CTx), and Osteocalcin (OC) was evaluated with kits. The tests were conducted thrice in accordance with the manufacturer's protocols (ELK Biotechnology, Wuhan, China).

Micro-CT evaluation

The Trabecular (Tb) bones were evaluated utilizing the Skyscan 1076 micro-CT device (Skyscan, Belgium). The collected bone specimens were subjected to a 70% ethanol solution and subsequently sectioned to a diameter of 21 µm. Subsequently, measures of Tb number, separation, thickness, Bone Volume/Total Volume (BV/TV), Structure Model Index (SMI), and Connectivity Density (CD) was evaluated.

Evaluation of calcium (Ca) homeostasis markers

The concentrations of Ca, Phosphorus (P), and 25-hydroxyvitamin-D (25-OH Vit-D) was investigated using kits. The tests were conducted thrice in accordance with the manufacturer's specifications (MyBioSource, USA).

Oxidative stress and inflammatory biomarker levels

The oxidative stress-related biomarkers, including Glutathione (GSH), Glutathione Peroxidase (GPx), Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Reductase (GR), and TBARS in both control and treated rats was assessed using assay kits. All assays were conducted in triplicate according to the manufacturer's guidelines (Elabscience, USA). Concentrations of pro-inflammatory cytokines interleukin-1β (IL-1β), IL-6, TNF-α, NF-κB, and Sirtuin 1 (SIRT1) were measured in the serum utilizing kits. The kits were procured from Elabscience, USA, and the tests were performed in triplicate following the directions specified in the kit brochure.

Analysis of bone remodeling marker protein levels

The serum levels of Osteoprotegerin (OPG), receptor activator of NF-κB Ligand (RANKL), runt-related transcription factor-2 (Runx-2), and Osterix (Osx) in rats was analyzed using commercially available test kits, adhering to the manufacturer's specifications (MyBioSource, USA).

Statistical analysis

The results were analyzed using GraphPad Prism, and the outcomes were illustrated as Mean±SD of triplicates. The one-way ANOVA was utilized for intergroup comparison, succeeded by Tukey's *post hoc* assay for multiple comparisons, with $p<0.05$ deemed as significant.

RESULTS

Effect of bilobetin on the biomechanical markers in experimental rats

The effects of bilobetin on biomechanical properties were evaluated in rats, with the results depicted in Figure 1. The rats with OVX-induced osteoporosis exhibited a marked reduction in biomechanical parameters, including maximum load, stress, stiffness, Young's modulus, and energy absorption measures in comparison to the sham-operated group. However, the bilobetin at a dose of 10 mg/kg effectively increased these biomechanical markers in OVX rats. The findings of bilobetin were further corroborated by the outcomes of alendronate.

Effect of bilobetin on bone turnover markers in experimental rats

The outcomes of the bilobetin treatment on the levels of bone turnover biomarkers are illustrated in Figure 2. The OVX-induced rats with osteoporosis exhibited a notable elevation in ALP, ACP, B-CTx, and OC concentrations. Whereas, the bilobetin at 10 mg/kg dosage displayed a notable reduction of these markers in OVX rats. The results are further corroborated by the alendronate, which also effectively diminishes these markers in OVA rats.

Effect of bilobetin on micro-CT analysis of Tb bone in experimental rats

The impact of bilobetin on micro-CT analysis of Tb bone in OVX rats was examined, and the findings are illustrated in Figure 3. The rats with OVX-induced osteoporosis exhibited a notable reduction in CD, BV/TV, Tb number, and Tb thickness, while demonstrating an elevation in SMI and Tb separation levels when compared with sham-operated controls. Notably, bilobetin at 10 mg/kg concentration elevated CD, BV/TV, Tb thickness, and Tb number, while decreasing SMI and Tb separation in OVX rats. The alendronate treatment also effectively modulated these alterations in the OVX rats, hence corroborating the efficacy of bilobetin.

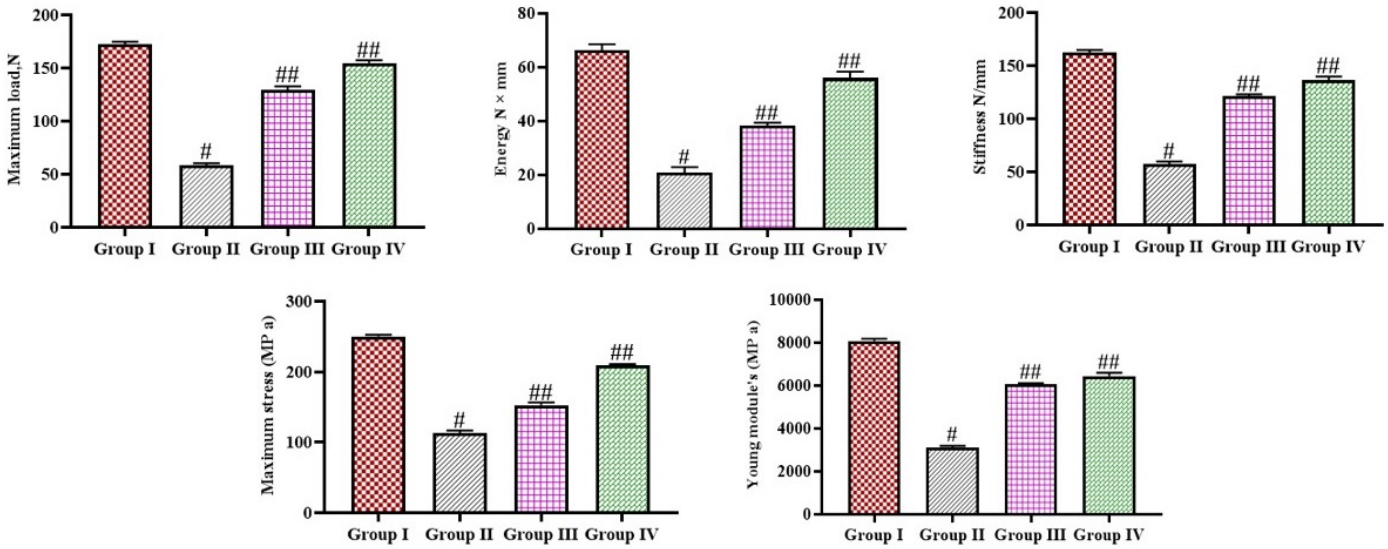

Figure 1: Effect of bilobetin on the biomechanical parameters in the experimental rats. The results were analyzed using GraphPad Prism, and the findings were expressed as Mean±SD of triplicates. The statistical significance for treatment groups were established as '#' $p<0.01$ for comparisons between the sham (group I) group and the OVX-induced osteoporosis group (Group II); '##' $p<0.05$ for comparisons between the OVX-induced group (Group II) and the OVX + bilobetin and/or alendronate-treated group (Group III/IV).

Effect of bilobetin on calcium homeostasis markers in experimental rats

Figure 4 illustrates the concentrations of Ca homeostasis markers in rats. It was observed that Ca and P concentrations were significantly increased, while 25-OH vit-D levels were diminished in OVX rats. Notably, the bilobetin at 10 mg/kg dosage effectively decreased Ca and P levels and enhanced 25-OH vit-D concentrations in OVX rats. Comparable outcomes were also observed with alendronate, which corroborates the efficacy of bilobetin.

Effect of bilobetin on oxidative stress biomarker levels in experimental rats

The effect of bilobetin administration on oxidative stress indicators in the experimental rats was depicted in Figure 5. The OVX-induced rats with osteoporosis demonstrated a significant decrease in GPx, GSH, SOD, GR, and CAT concentrations, alongside an elevation in TBARS levels than sham-operated controls. Interestingly, the 10 mg/kg of bilobetin treatment significantly elevated the concentrations of antioxidants while reducing TBARS levels in OVX rats.

Effect of bilobetin on inflammatory biomarkers in experimental rats

Figure 6 illustrates the concentrations of inflammatory biomarkers in rats. The rats with OVX-induced osteoporosis exhibited a substantial elevation in the SIRT1, TNF-α, IL-6, IL-1β, and NF-κB concentrations in relation to the sham-operated controls. Nevertheless, the administration of bilobetin at a 10 mg/kg concentration significantly diminished these inflammatory biomarkers in the OVX rats. These effects of bilobetin were further corroborated by the results of the alendronate, which also similarly decreased these cytokines in OVX rats.

Effect of bilobetin on bone remodeling marker proteins in experimental rats

The concentrations of bone remodeling marker proteins were quantified in the rats, with the results illustrated in Figure 7. The concentrations of Runx2, Osx, and OPG are significantly diminished; however, the RANKL is elevated in OVX rats with osteoporosis. Captivatingly, the treatment of bilobetin at a dose of 10 mg/kg remarkably elevated the levels of Osx, Runx2, and OPG, while concurrently reducing the RANKL level in the OVX rats.

DISCUSSION

Osteoporosis shows a considerable and increasing threat to public health worldwide, contributing substantially to morbidity, mortality, and socioeconomic burdens. The global prevalence of this debilitating skeletal disorder, where bone resorption surpasses bone formation, is increasing due to the aging global population, with projections indicating a substantial rise in the incidence of osteoporotic fractures and associated healthcare expenditures.[16] The resultant fragility fractures, particularly hip, vertebral, and wrist fractures, are associated with pain, disability, and decreased life quality. The risk of refracture is increased approximately twofold after any osteoporotic fracture, and this risk is even higher for men than women. Current pharmacological interventions for osteoporosis have been in decreasing fracture risk, but are also connected with potential side effects, like gastrointestinal disturbances, osteonecrosis, and atypical femoral fractures. The limitations and potential risks associated with current therapies underscore the urgent need to develop safe, potential, and affordable strategies to treat and

prevent osteoporosis.[17] The findings of the current study have evidenced the anti-osteoporotic efficacy of bilobetin against OVX-induced osteoporosis in the rat model.

OVX in rats serves as a well-established model for postmenopausal osteoporosis in humans, allowing researchers to investigate the pathogenesis of the disease and evaluate the potential of therapeutic agents. In this model, the surgical removal of the ovaries leads to estrogen deficiency, which in turn accelerates bone resorption and reduces bone development, leading to a net loss of bone mass and compromised bone microarchitecture.[18] The measurement of bone turnover biomarkers provides valuable knowledge into the dynamic processes of bone remodeling, reflecting the activity of osteoblasts and osteoclasts.[19] ALP is a widely used indicator of bone development, reflecting the osteoblast activity, the specialized cells committed to generating new bone matrix. Increased levels of ALP in serum indicate elevated osteoblast activity, which may be observed during periods of rapid bone growth or in response to bone injury or repair. Bone-specific ALP, an isoenzyme of ALP, is more specific to bone tissue and provides a more accurate assessment of osteoblast activity.[20] Bone-specific ALP is a tetrameric membrane-bound glycoprotein expressed by osteoblasts and participates in bone mineralization. Its activity is closely related to the rate of bone formation and can be utilized to observe the response to antiresorptive or anabolic therapies.[21] Furthermore, elevated levels of bone-specific ACP in serum indicate increased osteoclast activity and bone resorption. β-CTx, a fragment of type-I collagen, is another indicator of bone resorption. Type-I collagen is the predominant bone protein, and β-CTx is released into the bloodstream following the degradation of type-I collagen by osteoclasts. The determination of bone turnover markers like serum β-CTx enhances the ability to predict skeletal complications. Increased levels of β-CTx in serum are indicative of higher bone resorption.[22] OC, also known as bone Gla protein, is a non-collagenous protein generated by osteoblasts. OC is a vitamin K-dependent protein that binds to Ca and plays a role in bone mineralization. Measurements of OC levels in serum can provide information about osteoblast activity and bone formation rates. These biomarkers provide a comprehensive assessment of bone turnover, allowing researchers to differentiate between bone growth and resorption processes.[23] By monitoring changes in these markers in response to drug treatment, researchers can assess the efficacy of the drug in modulating bone remodeling. The findings of this work have demonstrated that OVX-induced rats exhibited a notable elevation of ALP, B-CTx, OC, and ACP concentrations. Though the bilobetin considerably decreased these bone turnover markers in the OVX rats.

Micro-CT analysis has emerged as a pivotal tool in preclinical bone research, offering unparalleled capabilities for the non-destructive assessment of Tb bone microarchitecture. Tb number, a direct

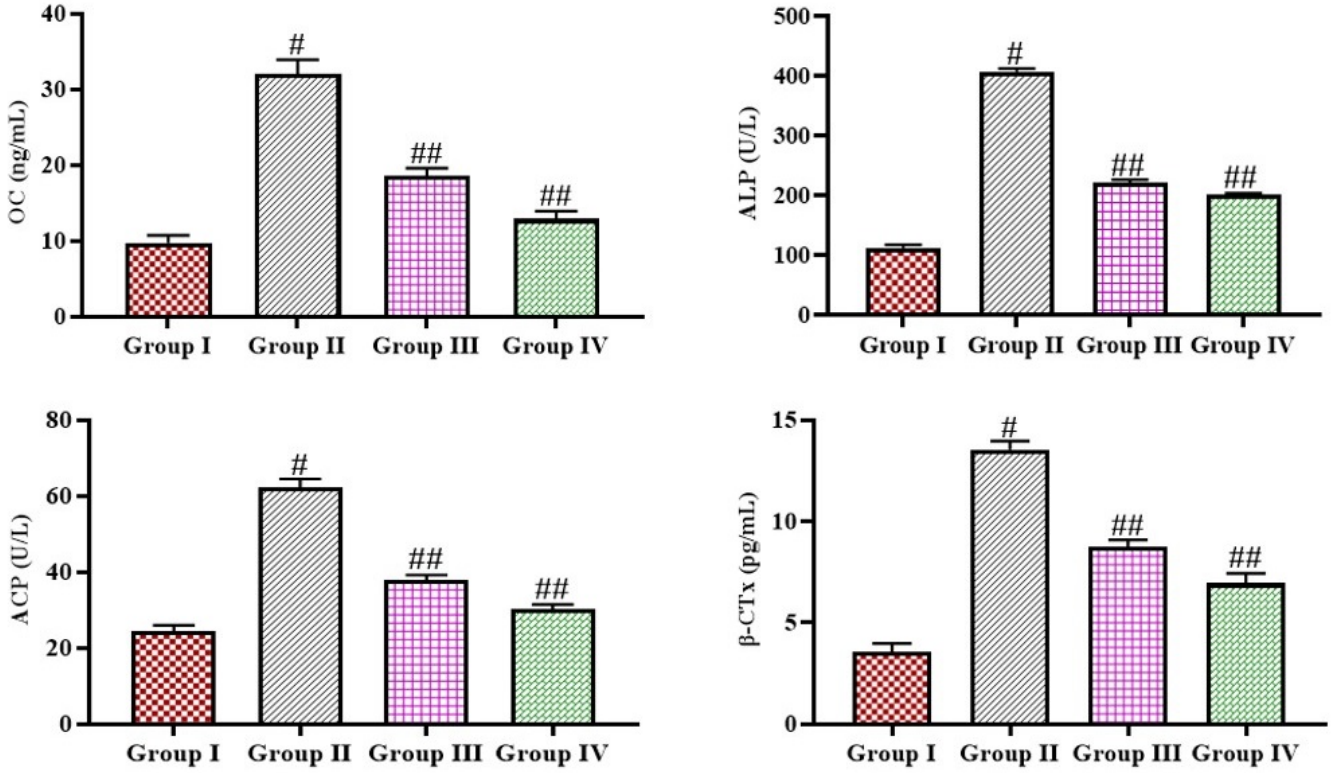

Figure 2: Effect of bilobetin on the bone turnover markers in the experimental rats. The results were analyzed using GraphPad Prism, and the findings were expressed as Mean±SD of triplicates. The statistical significance for treatment groups were established as '#' $p<0.01$ for comparisons between the sham (group I) group and the OVX-induced osteoporosis group (Group II); '##' $p<0.05$ for comparisons between the OVX-induced group (Group II) and the OVX + bilobetin and/or alendronate-treated group (Group III/IV).

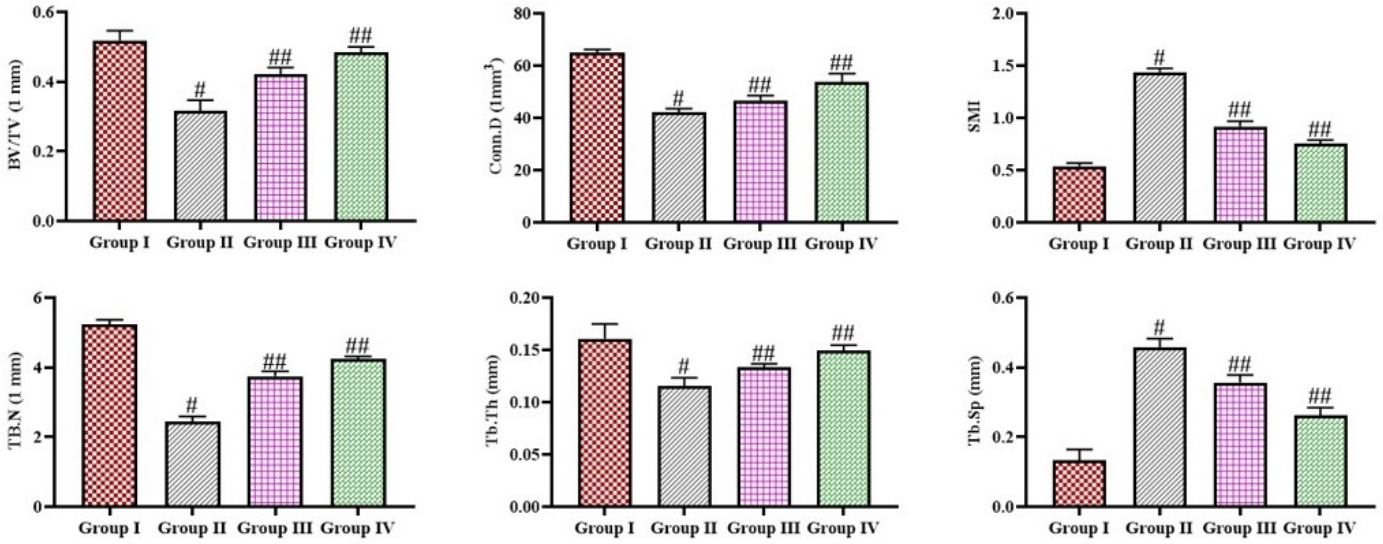

Figure 3: Effect of bilobetin on the micro CT analysis of Tb bone in the experimental rats. The results were analyzed using GraphPad Prism, and the findings were expressed as Mean±SD of triplicates. The statistical significance for treatment groups were established as '#' $p<0.01$ for comparisons between the sham (group I) group and the OVX-induced osteoporosis group (Group II); '##' $p<0.05$ for comparisons between the OVX-induced group (Group II) and the OVX + bilobetin and/or alendronate-treated group (Group III/IV).

measure of the density of Tb struts within the bone, is a critical parameter reflecting the structural integrity of cancellous bone. A higher Tb number typically indicates a greater capacity to withstand mechanical loads and a reduced susceptibility to fracture. Conversely, Tb separation, the inverse of the Tb number, quantifies the average distance between individual trabeculae. Increased Tb separation signifies a more porous and weakened bone structure, often associated with osteoporotic conditions.[24] Tb thickness, another essential microarchitectural parameter, represents the average width of individual trabeculae. Thicker trabeculae contribute to enhanced bone stiffness and resistance to compressive forces. Bone volume fraction (BV/TV), expressed as the ratio of bone volume to total volume, provides an overall assessment of bone density within a defined region of interest. A higher BV/TV ratio indicates a greater proportion of bone tissue, suggesting a healthier and more robust skeletal structure. Connectivity density, a sophisticated metric, quantifies the number of connections between Tb elements per unit volume. A higher connectivity density implies a more interconnected and mechanically resilient Tb network.[25] The SMI is an indicator that describes the plate-like or rod-like nature of Tb structures. A decreased SMI value indicates perfect plate-like structures, while an increased value indicates perfect rod-like structures. Increases in SMI suggest a shift from plate-like to rod-like Tb morphology, which is often associated with bone weakening.[26] The present findings indicated that the rats with OVX-induced osteoporosis illustrated reduced CD, BV/TV, Tb number, and Tb thickness, while showing an elevated SMI and Tb separation. Interestingly, the bilobetin treatment effectively augmented CD, BV/TV, Tb thickness, and Tb number while decreasing the SMI and Tb separation in OVX rats.

The pathogenesis of osteoporosis is multifactorial, with Ca homeostasis playing a pivotal role in maintaining bone integrity. The significance of Ca homeostasis in osteoporosis extends beyond bone mineralization, encompassing intricate cellular signaling pathways that govern osteoblast and osteoclast activity.[27] Ca, P, and 25-OH Vit-D serve as crucial indicators of Ca homeostasis, offering valuable insights into the onset of osteoporosis. Ca, the primary mineral constituent of bone, dictates bone strength and rigidity; its homeostasis is governed by an intricate interplay between intestinal absorption, renal excretion, and bone remodeling. P, another essential mineral, collaborates with Ca to fortify the bone matrix, while 25-OH Vit-D, a precursor to the active form of vitamin D, plays a vital role in Ca absorption and bone metabolism.[28] Derangements in Ca homeostasis, as reflected by fluctuations in serum Ca, P, and 25-OH Vit-D levels, can disrupt bone remodeling, predisposing individuals to osteoporosis. Vitamin D deficiency, a widespread concern, hampers Ca absorption, triggering compensatory mechanisms such as increased parathyroid hormone secretion, which in turn accelerates bone resorption. The intricate relationship between Ca, P, and vitamin D underscores the necessity of comprehensive monitoring and management of these markers in individuals at risk of osteoporosis.[29] In this work, we found that the Ca and P concentrations were increased, while 25-OH vit-D concentrations were diminished in OVX rats. Interestingly, the bilobetin treatment markedly reduced Ca and P levels and augmented 25-OH vit-D concentrations in the OVX rats. Therefore, it was clear that bilobetin effectively regulated the Ca homeostasis in osteoporosis-induced rats.

The pathophysiology of osteoporosis is multifaceted, involving a complex interplay of genetic, hormonal, and environmental factors. While the imbalance between bone formation by

osteoblasts and bone resorption by osteoclasts is a well-established hallmark of osteoporosis, oxidative stress is a crucial contributor to its development and progression. Oxidative stress arises from the disproportion of ROS generation and the capacity of the antioxidant defense to neutralize them. ROS, while essential for certain physiological processes such as osteoclast differentiation, can become detrimental when their levels overwhelm the antioxidant defenses, leading to cellular damage and participating in the onset of several diseases, including osteoporosis.[30] Oxidative stress affects Tb bone score, bone turnover markers, and increases bone fractures. While the maintenance of redox homeostasis is essential for proper cellular function, an excess of ROS can overwhelm the endogenous antioxidant systems, resulting in oxidative injury to the cell components, thus contributing to the pathophysiology of osteoporosis. Estrogen decline during menopause diminishes the antioxidant defense mechanisms, increasing oxidative stress that affects bone remodeling.[31]

GSH, a tripeptide, is a crucial part of the cellular antioxidant defense, playing a direct role in neutralizing ROS and acting as a cofactor for various antioxidants. GPx, a selenium-containing enzyme, induces the reduction of hydrogen and lipid peroxides, thereby protecting cells from oxidative injury. SOD, another essential antioxidant, induces the dismutation of superoxide radicals into hydrogen peroxide and oxygen, representing a crucial step in the detoxification of ROS.[32] CAT, a heme-containing enzyme, facilitates the decomposition of hydrogen peroxide into water and oxygen, further mitigating the damaging effects of ROS. GR, a flavoprotein enzyme, maintains the reduced form of GSH, ensuring the continued functionality of the GSH-dependent antioxidant system.[33] TBARS are a group of compounds produced during lipid peroxidation, a chain reaction initiated by ROS that results in the degradation of lipids, leading to membrane damage and cell dysfunction. The measurement of TBARS is widely used as a marker of oxidative stress and lipid peroxidation in several biological systems.[34] The study of oxidative stress markers offers valuable insights into the mechanisms underlying osteoporosis and may offer potential therapeutic targets to treat and prevent osteoporosis. The current findings exhibited that the OVX-induced rats showed decreased GSH, GPx, SOD, GR, and CAT levels, alongside with increased TBARS levels. However, the bilobetin treatment significantly elevated the antioxidant levels while reducing TBARS levels in the OVX rats, which suggests its antioxidant effects.

Inflammation, orchestrated by a complex network of immune cells and signaling molecules, exerts its influence on bone metabolism through the action of pro-inflammatory cytokines. Cytokines are

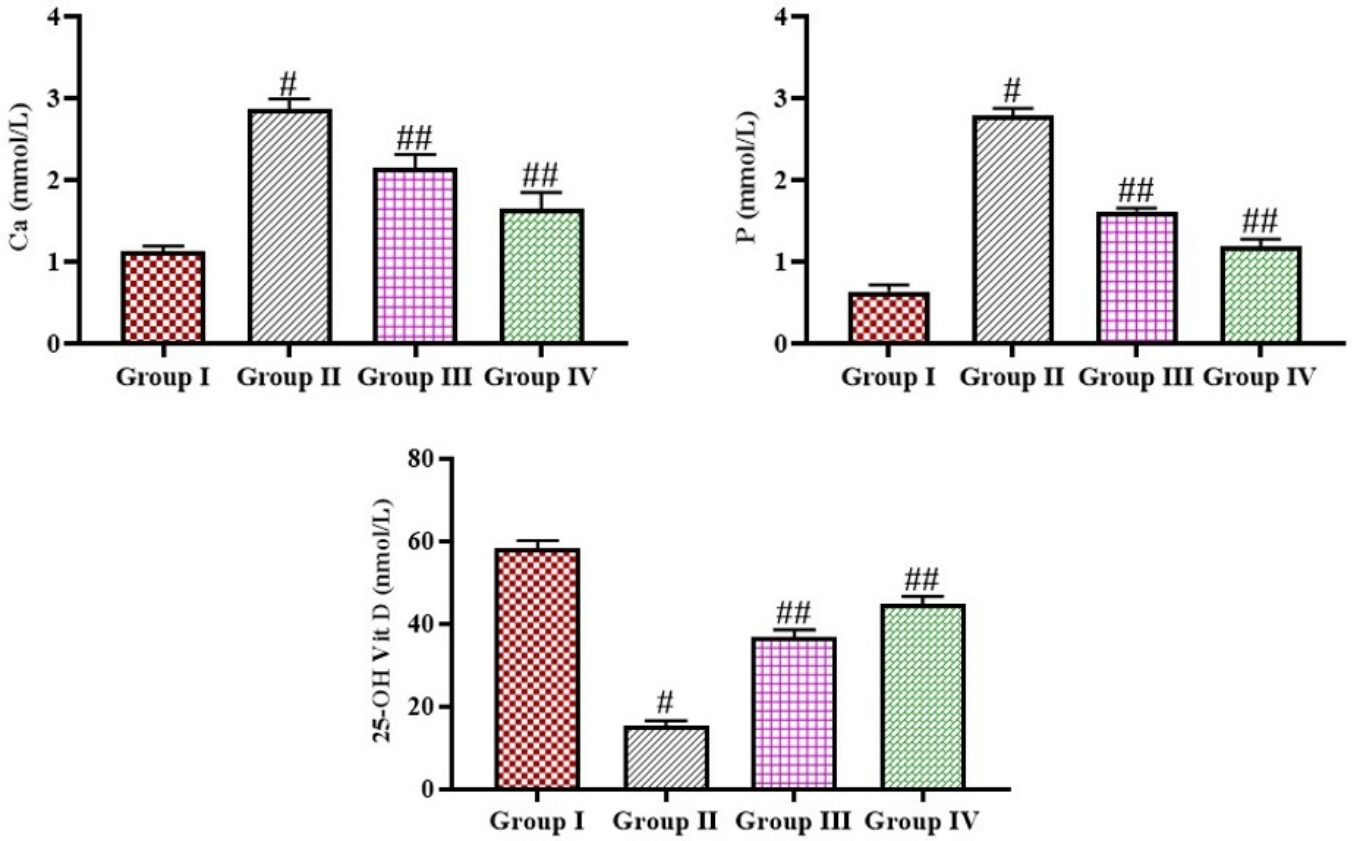

Figure 4: Effect of bilobetin on the Ca homeostasis markers in the experimental rats. The results were analyzed using GraphPad Prism, and the findings were expressed as Mean±SD of triplicates. The statistical significance for treatment groups were established as '#' $p<0.01$ for comparisons between the sham (group I) group and the OVX-induced osteoporosis group (Group II); '##' $p<0.05$ for comparisons between the OVX-induced group (Group II) and the OVX + bilobetin and/or alendronate-treated group (Group III/IV).

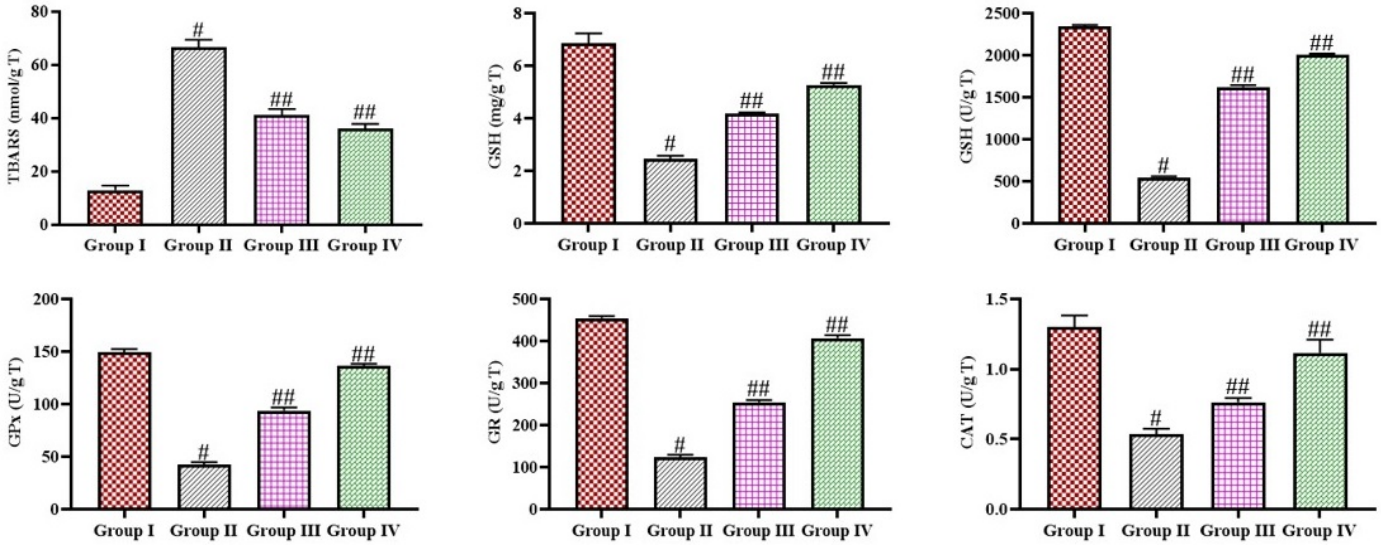

Figure 5: Effect of bilobetin on the oxidative stress marker levels in the experimental rats. The results were analyzed using GraphPad Prism, and the findings were expressed as Mean±SD of triplicates. The statistical significance for treatment groups were established as '#' $p<0.01$ for comparisons between the sham (group I) group and the OVX-induced osteoporosis group (Group II); '##' $p<0.05$ for comparisons between the OVX-induced group (Group II) and the OVX + bilobetin and/or alendronate-treated group (Group III/IV).

essential in bone remodeling, which is a pathological process in the onset of osteoporosis. IL-1β, IL-6, and TNF-α are essential inflammatory cytokines implicated in the pathogenesis of osteoporosis. These cytokines, released by immune cells and other cell types within the bone microenvironment, have the capacity to directly and indirectly modulate the activity of both osteoclasts and osteoblasts, thereby disrupting the delicate equilibrium of bone remodeling.[35] The impact of inflammation and pro-inflammatory cytokines is significant in initiating and maintaining osteoclastogenesis, which ultimately leads to bone resorption by osteoclasts. IL-1β, an essential cytokine, plays an imperative role in the pathogenesis of osteoporosis by stimulating osteoclastogenesis and inhibiting osteoblast activity. Furthermore, IL-1β can amplify the generation of NF-κB ligand by osteoblasts and stromal cells, further promoting osteoclast formation and activation.[36] Additionally, IL-1β has been shown to reduce the differentiation and matrix synthesis of osteoblasts, impairing bone development and contributing to the overall bone loss in osteoporosis. The capacity of IL-1β to simultaneously stimulate bone resorption and inhibit bone formation underscores its importance as a critical regulator of inflammation-induced bone loss.[37]

IL-6, another prominent pro-inflammatory cytokine, exerts its influence on bone metabolism through multiple mechanisms, contributing to the pathogenesis of osteoporosis. Furthermore, IL-6 can trigger the generation of other inflammatory cytokines, creating a positive feedback loop that amplifies the inflammatory response and its detrimental effects on bone. It is noteworthy that elevated levels of IL-6 are observed in postmenopausal women and are correlated with decreased BMD, suggesting that IL-6 plays an essential role in the onset of postmenopausal osteoporosis.[38] TNF-α, a pleiotropic cytokine with diverse biological activities, is an essential regulator of inflammation-mediated bone loss in osteoporosis. TNF-α directly stimulates osteoclastogenesis, triggering the activation of intracellular signaling that enhances their differentiation and activity. TNF-α also improves the generation of other cytokines, amplifying the inflammatory cascade and its detrimental effects on bone. Additionally, TNF-α can inhibit osteoblast differentiation and function, impairing bone growth and contributing to the overall bone loss in osteoporosis.[39]

NF-κB, a transcription factor that controls the expression of several genes participated in inflammation, immunity, and cell survival, plays a central role in the pathogenesis of osteoporosis. NF-κB activation is a central event in RANKL-induced osteoclastogenesis. In osteoclasts, NF-κB activation promotes their differentiation, survival, and activity, leading to increased bone resorption. NF-κB also regulates the expression of several cytokines, developing a positive feedback loop that enhances the inflammation and its detrimental effects on bone. Furthermore, NF-κB has been shown to inhibit osteoblast differentiation and function, impairing bone growth and contributing to the overall bone loss in osteoporosis.[40] SIRT1, a highly conserved NAD+-dependent deacetylase, has developed as an essential mediator of aging and age-related diseases, including osteoporosis. SIRT1 was shown to exert protective activity on bone by modulating various cellular processes involved in bone remodeling. SIRT1 can deacetylate and activate several transcription factors and signaling molecules that promote osteoblast activity and bone growth. SIRT1 promotes osteoblast differentiation by deacetylating and activating Runx2, a master transcription factor that controls the gene expression essential for osteoblast development and function.[41] The current findings suggested the elevated TNF-α, SIRT1, NF-κB, IL-6, and IL-1β in the rats with

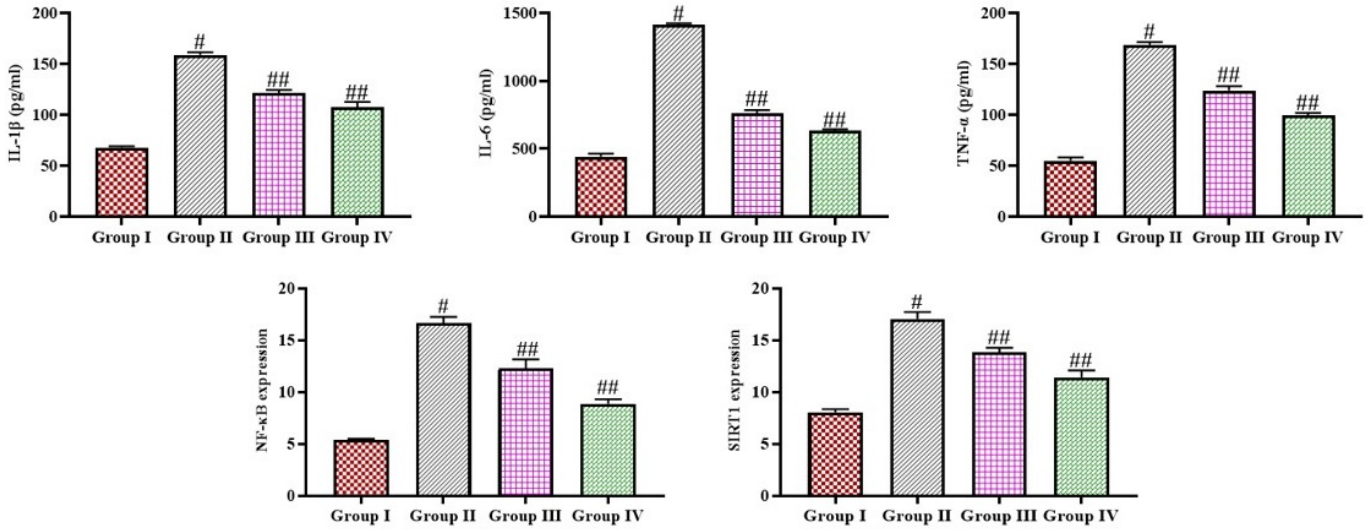

Figure 6: Effect of bilobetin on the inflammatory biomarker levels in the experimental rats. The results were analyzed using GraphPad Prism, and the findings were expressed as Mean±SD of triplicates. The statistical significance for treatment groups were established as '#' $p<0.01$ for comparisons between the sham (group I) group and the OVX-induced osteoporosis group (Group II); '##' $p<0.05$ for comparisons between the OVX-induced group (Group II) and the OVX + bilobetin and/or alendronate-treated group (Group III/IV).

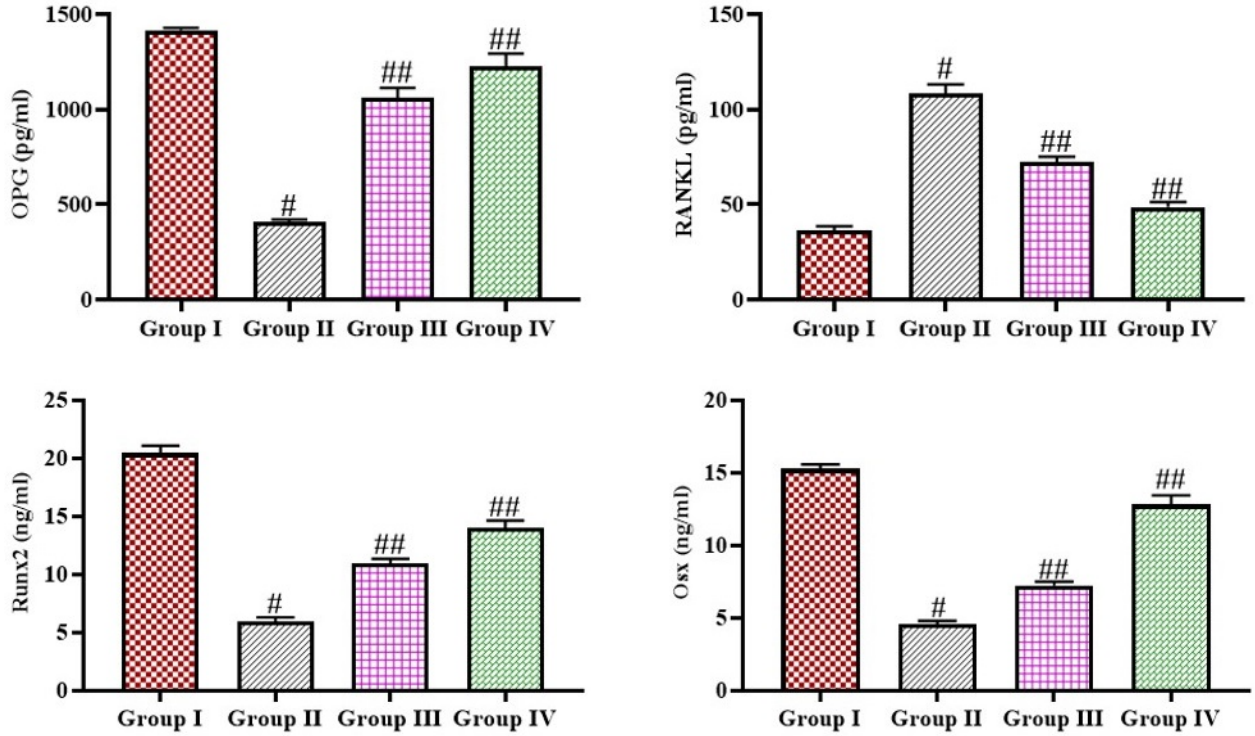

Figure 7: Effect of bilobetin on the bone remodeling marker protein levels in the experimental rats. The results were analyzed using GraphPad Prism, and the findings were expressed as Mean±SD of triplicates. The statistical significance for treatment groups were established as '#' $p<0.01$ for comparisons between the sham (group I) group and the OVX-induced osteoporosis group (Group II); '*' $p<0.05$ for comparisons between the OVX-induced group (Group II) and the OVX + bilobetin and/or alendronate-treated group (Group III/IV).

OVX-induced osteoporosis. Nonetheless, the bilobetin treatment markedly reduced these inflammatory biomarkers in the OVX rats that supports its anti-inflammatory activity.

Several key molecular players, including OPG, RANKL, Osx, and Runx2, play pivotal roles in regulating bone remodeling and are implicated in the onset of osteoporosis. The disruption of this equilibrium, where bone resorption exceeds bone development, results in decreased BMD and microarchitectural deterioration, the hallmarks of osteoporosis.[42] OPG, a soluble decoy receptor of the TNF superfamily, serves as a critical mediator of osteoclastogenesis, the formation of osteoclasts. OPG functions by binding to RANKL, a transmembrane protein expressed by osteoblasts and stromal cells, thus averting RANKL from interacting with its receptor RANK, which is expressed on osteoclast precursors.[43] This interaction blocks osteoclast

differentiation, fusion, and activation, effectively suppressing bone resorption. By neutralizing RANKL, OPG effectively reduces the stimulus for osteoclast formation and activity, thereby shifting the balance towards bone formation.[44] Runx2 plays a key role in bone growth and remodeling. Runx2 mediates several gene expressions involved in osteoblast differentiation.[45] Osx is a zinc-finger-containing transcription factor that acts downstream of Runx2 in the osteoblast differentiation pathway. Osx is specifically expressed in osteoblasts, and it is crucial for the terminal differentiation of osteoblasts and bone growth. Osx regulates gene expression, participates in bone matrix synthesis, and mineralization.[46] The interplay between OPG, RANKL, Runx2, and Osx is critical for maintaining bone homeostasis, and disruptions in this delicate balance contribute to the onset of osteoporosis.[47] The current findings exhibited that the concentrations of Runx2, OPG, and Osx are significantly diminished along with elevated RANKL in the OVX rats. Interestingly, the bilobetin considerably elevated the Runx2, Osx, and OPG concentrations, while reducing the RANKL in OVX rats. These findings highlighted that bilobetin effectively regulated the bone remodeling proteins in the OVX-induced rats with osteoporosis.

CONCLUSION

The present findings elucidated the anti-osteoporosis effects of bilobetin against OVX-induced rats. The findings of this study indicated that bilobetin treatment restored bone turnover marker levels, mediated Ca homeostasis, improved biomechanical properties, diminished inflammatory cytokines, mitigated oxidative stress via elevating antioxidants, augmented OPG and Runx2 concentrations, and reduced RANKL levels in OVX rats. These data indicated the advantageous effects of bilobetin in mitigating osteoporosis in OVX rats. However, further studies are necessary to provide further evidence on the anti-osteoporosis characteristics of bilobetin, thereby establishing it as a promising pharmacological treatment for osteoporosis management.

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ABBREVIATIONS

OVX: Overiectomy; **Ca:** Calcium; **ALP:** Alkaline Phosphatase; **β-CTx:** Beta-CrossLaps; **OC:** Osteocalcin; **Tb:** Trabecular; **SMI:** Structure Model Index; **CD:** Connectivity Density; **GPx:** Glutathione Peroxidase; **CAT:** Catalase; **IL:** Interleukin; **SIRT1:** Sirtuin 1; **OPG:** Osteoprotegerin; **RANKL:** Receptor activator of NF-κB Ligand; **Runx-2:** Runt-related transcription factor-2; **Osx:** Osterix.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL STATEMENT

This work was approved by the IEAC of The Second Affiliated Hospital of Guangdong Medical University, 524003, Guangdong Province, China.

SUMMARY

Osteoporosis is a significant risk to global public health, substantially contributing to morbidity, mortality, and socioeconomic costs. The OVX rat model is a recognized system for studying postmenopausal osteoporosis in humans, enabling researchers to explore the disease's etiology and assess the efficacy of prospective treatment therapies. The current findings clarified the anti-osteoporosis effects of bilobetin in OVX rats. This study's findings demonstrated that bilobetin treatment restored bone turnover markers, regulated calcium homeostasis, enhanced biomechanical properties, decreased inflammatory cytokines, alleviated oxidative stress by increasing antioxidants, elevated OPG/Runx2 concentrations, and lowered RANKL levels in OVX rats. The data demonstrated the beneficial effects of bilobetin in alleviating osteoporosis in OVX rats.

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