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Antiosteoporosis and bone protective effect of alpinumisoflavone in steroid-induced osteoporosis rats *via* alteration of apoptosis, inflammatory and RANK/RANKL/OPG signaling pathway

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ABSTRACT

Objective: To estimate the bone protective effect of alpinumisoflavone, a natural prenylated isoflavonoid, against glucocorticoid-induced osteoporosis in rats.

Methods: Male Wistar rats received intramuscular administration of dexamethasone (4 mg/mL) at a dose level of 7 mg/kg for 5 weeks, and then alpinumisoflavone (5, 10, and 15 mg/kg) and alendronate (2 mg/kg) from 2 weeks. The body weight and organ weight (femoral, vagina, and uterus) were estimated. MicroCT analysis, bone turnover markers, bone parameters, oxidative stress parameters, and inflammatory cytokines were estimated. mRNA expressions of related genes were also estimated.

Results: Alpinumisoflavone remarkably boosted body weight and organ weight (ureters and vagina), improved microCT analysis parameters, and boosted levels of bone markers. Besides, alpinumisoflavone considerably (P<0.001) restored the level of bone turnover markers and oxidative stress parameters, remarkably suppressed the level of cytokines such as interleukin-1β, interleukin-6, tumor necrosis factor-α, and increased transforming growth factor-β and insulin-like growth factor. It also significantly restored the osteoprotegerin (OPG, RANKL, and OPG/RANKL ratio) levels and the mRNA expression of *Caspase* (3, 6, 7, 9), *BMP*s, *OPN*, *ALP*, *RUNX2*, and *OCN*.

Conclusions: The current result suggests the bone protective effect of alpinumisoflavone against glucocorticoid-induced osteoporosis in rats.

KEYWORDS: Osteoporosis; Alpinumisoflavone; Bone parameters; Inflammation; Oxidative stress

1. Introduction

Osteoporosis disease mostly hits middle-aged and elderly individuals. The main characteristic features of osteoporosis are the loss of bone and dysregulation of bone tissue structure, which further induces metabolic dysfunctions[1]. As per the reports, the incidence of osteoporosis increases globally day by day, and approximately 200 million people suffer from the disease.

Significance

These findings suggest that alpinumisoflavone could be a promising candidate for the prevention or treatment of glucocorticoid-induced osteoporosis. The ability of alpinumisoflavone to modulate various biochemical and molecular pathways involved in bone health underscores its therapeutic potential. This research contributes to the growing body of evidence supporting the development of novel treatments for osteoporosis and highlights the importance of natural compounds in managing bone health.

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Osteoporosis disease increases the socioeconomic burden due to the high rate of dysfunction and morbidity, and sometimes, it causes death[2]. Osteoblasts and osteoclasts work in tandem to regulate the equilibrium between bone formation and bone breakdown. Glucocorticoids disrupt this delicate balance, leading to osteoporosis by skewing the ratio between bone formation and resorption.

Glucocorticoid-induced osteoporosis (GIOP) stands out as one of the most common forms of secondary osteoporosis. Glucocorticoids are extensively utilized to treat a range of medical conditions, such as inflammatory diseases, organ transplantation, autoimmune disorders, and diverse cancer types[3]. Glucocorticoids can negatively impact bone health by disturbing the delicate equilibrium between bone formation and resorption. These medications influence various pathways in bone metabolism, resulting in reduced bone formation, heightened bone resorption, and impaired calcium absorption. This disruption in bone remodeling processes can lead to weakened bones and an increased susceptibility to fractures[4,5]. Glucocorticoids such as dexamethasone (DEX), cortisone, or prednisone were used for the induction of osteoporosis as a high-dose or long-term treatment. Indeed, there are additional factors that contribute to the development of GIOP. These factors include the underlying disease or condition being treated with glucocorticoids and various lifestyle-related elements[6,7]. The underlying disease or condition can contribute to bone loss and increase the risk of osteoporosis when treated with glucocorticoids over the long term. Maintaining bone health requires lifestyle changes like ensuring an appropriate calcium and vitamin D diet, regular weight-bearing activity, and abstaining from smoking and binge drinking[8,9].

Oxidative stress indeed plays a pivotal role in developing age-related bone loss. The boosted level of oxidative stress shows the reduced level of antioxidant enzymes like catalase (CAT) and glutathione peroxidase (GPx) in osteoporotic patients[10]. Therefore, enhanced oxidative stress is accelerated by enhancing the duration of the glucocorticoid treatment[11]. According to previous reports, it has been suggested that DEX can induce oxidative stress through either direct or indirect mechanisms. Oxidative stress can result from increased production of reactive oxygen species (ROS) or decreased activity of endogenous antioxidant enzymes[11,12]. In either case, DEX can potentially disrupt the delicate balance between oxidants and antioxidants in the body, leading to an imbalance favoring oxidative stress[13]. Nuclear factor erythroid 2-related factor 2 (Nrf2) plays a protective role against osteoporosis by upregulating the expression of antioxidant enzymes and reducing oxidative stress. So, it is more accurate to say that Nrf2 helps mitigate osteoporosis by decreasing the production of ROS and enhancing antioxidant defenses. Mitochondrial dysfunction suppresses bone mineralization, boosting osteocyte apoptosis *via* ROS overproduction[11,13]. By leveraging the antioxidant-responsive element in conjunction with an Nrf2 activator, the progression of osteoporosis is hindered through the augmentation of antioxidant enzyme expression levels[14]. Indeed, the Nrf2 pathway is recognized for its regulation of osteoblast activity. The glucocorticoid treatment boosts the inflammatory cytokine levels. Also, it boosts the chance of fracture incidence and bone loss *via* exerting the direct effect on signaling molecules of peroxisome proliferator-activated receptor gamma receptor 2[15], induction of apoptosis in osteocyte, reduction of Wnt/β-catenin signaling pathway in osteoblast[16], receptor activator of nuclear factor kappa-B ligand in osteoclast[17] and stimulation of macrophage colony-stimulating factor[16].

Over the past few decades, alternative medicines have displayed promising potential as a viable option for addressing the limitations associated with conventional osteoporosis treatments. Alpinumisoflavone is a natural compound found in various plant species. It belongs to a class of compounds known as coumarins. Alpinumisoflavone has been studied for its potential therapeutic effects, including its anti-inflammatory, antioxidant, and anticancer properties. Research on the potential therapeutic benefits of the Nrf2 pathway in treating osteoporosis is ongoing. Preclinical studies have demonstrated promising results, showing its ability to inhibit bone loss and promote bone formation. However, before it can be widely adopted as a treatment for osteoporosis, additional research must fully elucidate its mechanisms of action and assess its efficacy and safety in human subjects. In this study, we investigated the anti-osteoporosis effect of alpinumisoflavone against DEX-induced osteoporosis in rats and explored the underlying mechanisms.

2. Materials and methods

2.1. Experimental rodent

Sprague Dawley rats [10-12 weeks old, (135±20) g, male] were used in this experimental study. The whole animal study was carried out according to the Hospital's Ethics Committee principles for the care and use of experimental animals. The rats were kept in ventilated cages and maintained in standard conditions [(22±5)℃, 60%-70% relative humidity, and 12 h/12 h light and dark cycle]. The rats received deionized water and free access to food. Male rats were chosen for this study to avoid potential confounding factors related to the hormonal fluctuations observed in female rats during the estrous cycle, which could have impacted the experimental outcomes and results.

2.2. Rodent diet and ethical statement

The rats were provided with a purified, balanced diet formulated for maintaining adult rats, following the specifications of Reeves *et al*.[18]. The rats underwent a one-week acclimatization period to adjust to the laboratory conditions before the commencement of the experimental procedures. The whole study was approved by the Jiangsu University (2024No.10).

2.3. GIOP rat model

Following the acclimatization period, the rats were divided into groups as follows.

Group A: Normal control, Group B: DEX (7 mg/kg), Group C: DEX (7 mg/kg) + alpinumisoflavone (5 mg/kg), Group D: DEX (7 mg/kg) + alpinumisoflavone (10 mg/kg), Group E: DEX (7 mg/kg) + alpinumisoflavone (15 mg/kg), and Group F: DEX (7 mg/kg) + alendronate (2 mg/kg), respectively.

They then received intramuscular administration of DEX (4 mg/mL) at a dose level of 7 mg/kg for 5 weeks[19]. After administering DEX for 2 weeks, the rats were administered alpinumisoflavone orally. Throughout the experimental period, the rats' water intake, food consumption, and body weight were monitored weekly.

2.4. Sample collection

At the end of the experimental study, the rats were euthanized under anesthesia. Blood samples were obtained by puncturing the retro-orbital plexus under anesthesia and centrifuging at 10000 rpm for 5 min to separate the serum. The serum was then collected and stored at –20℃ for further analysis.

Additionally, tissue samples for additional biochemical markers were collected after euthanasia under anesthesia and preserved at –80℃ for subsequent analysis.

2.5. Organ weight

A previously reported method was used to estimate organ weight[11,19]. The organs, such as the femur, vagina, and uterus, were removed after sacrificing the rats and weighed before drying at 65℃ to ensure an invariable weight.

2.6. MicroCT analysis

The right tibias were removed right away after the rats were sacrificed. The bone tissue was adequately preserved, fixed, and scanned in sample cups. The study chose the tibia's diaphyseal and inflated region of interest after successfully scanning it. The region of interest of the specimens was rebuilt after the threshold values for three-dimensional reconstruction were selected. The total volume (TV), trabecular number (Tb.N), bone surface/bone volume (BS/BV), trabecula thickness (Tb.Th), trabecula separation (Tb.Sp), and bone volume/total volume (BV/TV) were all examined.

2.7. Bone turnover markers

Bone turnover markers, including bone-specific alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (TRAP), acid phosphatase (ACP), and bone Gla protein (BGP) were assessed using commercially available kits from Nanjing Jiancheng Bioengineering Institute, China.

The β-isomerized C-terminal telopeptide of type I collagen (β-CTX) and osteocalcin (OCN) were estimated using the available commercial kits following the manufacturer's instructions (USCN Life Science, China).

2.8. Bone parameters

Bone parameters such as bone mineral content (BMC), bone mineral density (BMD), tissue mineralized content (TMC), and tissue mineralized density (TMD) were estimated using the previous report procedure with the help of Lunar Prodigy Dpxa-IQ-7040 (GE Healthcare, USA). The level of bone parameters was estimated using the DEXA built-in software package.

2.9. Oxidative stress parameters

Oxidative stress parameters, including thiobarbituric acid reactive substances (TBARS), glutathione-S-transferase (GST), CAT, superoxide dismutase (SOD), glutathione (GSH), GPx, and glutathione reductase (GR), were measured according to the manufacturer's instructions using ELISA kits from Nanjing Jiancheng Bioengineering Institute, China.

2.10. Cytokines

The cytokine parameters like tumor necrosis factor-α (TNF-α), interleukin (IL)-1β (IL-1β), IL-6, insulin-like growth factor (IGF), and transforming growth factor-β (TGF-β), as well as osteoprotegerin (OPG) and RANKL were estimated according to the manufacturer's instruction of ELISA kits (Nanjing Jiancheng Bioengineering Institute, China).

2.11. Reverse transcription–quantitative real–time polymerase chain reaction (RT–qPCR)

RNAisol Plus reagent was used to isolate total RNA from the rodent, following the manufacturer's instructions. ND1000 spectrophotometer was used to quantify the total RNA concentration at different wavelengths. The total RNA (1 mg) synthesized the first strand of cDNA following the manufacturer's instructions (Takara Bio Inc.) with appropriate reverse and forward primers. The set of primers is presented in Supplementary Table 1. It was performed at 94℃ for 15 s, 40 cycles, 55℃ for 30 s, and 72℃ for 30 s. All the reaction was carried out in triplicate, and the result was analyzed *via* the $2^{-\Delta\Delta CT}$ method, and *β-actin* was used as an internal standard gene.

2.12. Statistical analysis

All data were analyzed using GraphPad Prism software (St. Louis, USA). Statistical comparisons among different groups were

performed using one-way ANOVA followed by Dunnett's *t*-test. Results are presented as mean ± standard error of the mean (SEM), with significance as $P<0.05$.

3. Results

3.1. Body weight and organ weight

The body weight decreased during the osteoporosis disease. GIOP group rats showed reduced body weight (Figure 1A) and exhibited an alteration in the weight of femoral (Figure 1B), vagina (Figure 1C), and uterus weight (Figure 1D). Alpinumisoflavone significantly ($P<0.01$) increased the body weight and improved the vagina and uterus weight. The femoral weight of all group rats remained unchanged.

3.2. MicroCT analysis

MicroCT parameters are used to analyze the microarchitecture of trabecular bone, which is the spongy inner part of bone tissue. Figure 2 exhibits the altered level of microCT parameters in different groups. GIOP rats exhibited the altered level of Tb.N (Figure 2A), Tb.Th (Figure 2B), BV/TV (Figure 2C), BS/BV (Figure 2D), and Tb.Sp (Figure 2E). Alpinumisoflavone improved the level of microCT parameters in a dose-dependent manner.

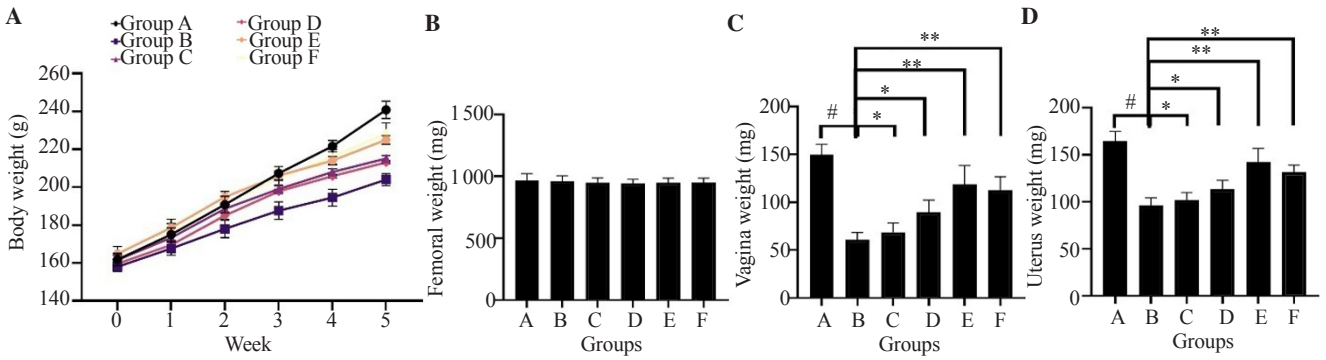


Figure 1. Effect of alpinumisoflavone on body weight and organ weight. A: body weight, B: femoral weight, C: vagina weight, and D: uterus weight. The whole data are shown as mean ± SEM. $^{\#}P<0.001$ as compared with normal control (Group A), $^{*}P<0.05$, $^{**}P<0.001$ as compared to glucocorticoid-induced osteoporosis (GIOP) group (Group B). Dunnett's *post hoc* test following One-Way Analysis of Variance (ANOVA). Group A: Normal control, Group B: GIOP group, Group C: GIOP+alpinumisoflavone (5 mg/kg), Group D: GIOP+alpinumisoflavone (10 mg/kg), Group E: GIOP+alpinumisoflavone (15 mg/kg), Group F: GIOP+alendronate (2 mg/kg).

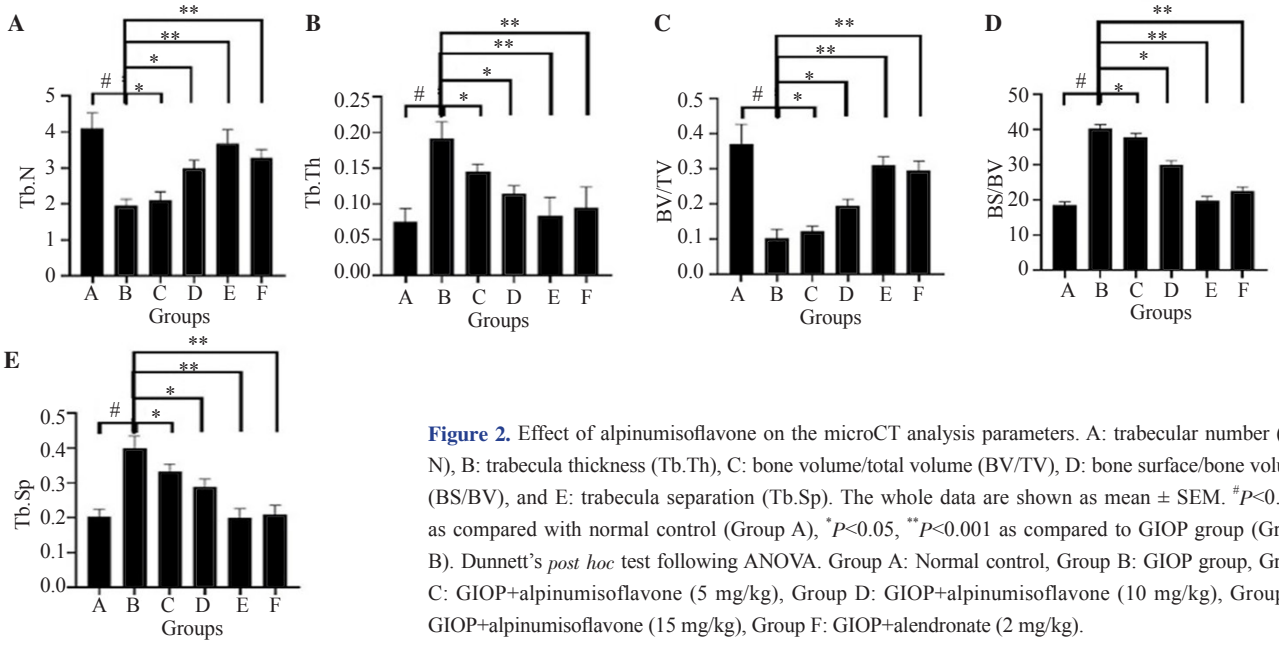


Figure 2. Effect of alpinumisoflavone on the microCT analysis parameters. A: trabecular number (Tb.N), B: trabecula thickness (Tb.Th), C: bone volume/total volume (BV/TV), D: bone surface/bone volume (BS/BV), and E: trabecula separation (Tb.Sp). The whole data are shown as mean ± SEM. $^{\#}P<0.001$ as compared with normal control (Group A), $^{*}P<0.05$, $^{**}P<0.001$ as compared to GIOP group (Group B). Dunnett's *post hoc* test following ANOVA. Group A: Normal control, Group B: GIOP group, Group C: GIOP+alpinumisoflavone (5 mg/kg), Group D: GIOP+alpinumisoflavone (10 mg/kg), Group E: GIOP+alpinumisoflavone (15 mg/kg), Group F: GIOP+alendronate (2 mg/kg).

3.3. Bone parameters

Bone parameters are related to the measurement of bone density and are commonly used in the diagnosis and assessment of osteoporosis. GIOP group rats exhibited suppressed levels of BMD (Figure 3A), TMD (Figure 3B), TMC (Figure 3C), and BMC (Figure 3D), while alpinumisoflavone treatment significantly (P<0.05) enhanced the level of bone parameters.

3.4. Serum levels of bone turnover markers

GIOP group rats displayed decreased levels of OCN (Figure 4A), elevated levels of TRAP (Figure 4B), β-CTX (Figure 4C), BGP (Figure 4D), ACP (Figure 4E), and ALP (Figure 4F), while alpinumisoflavone significantly (P<0.05) restored levels of these parameters.

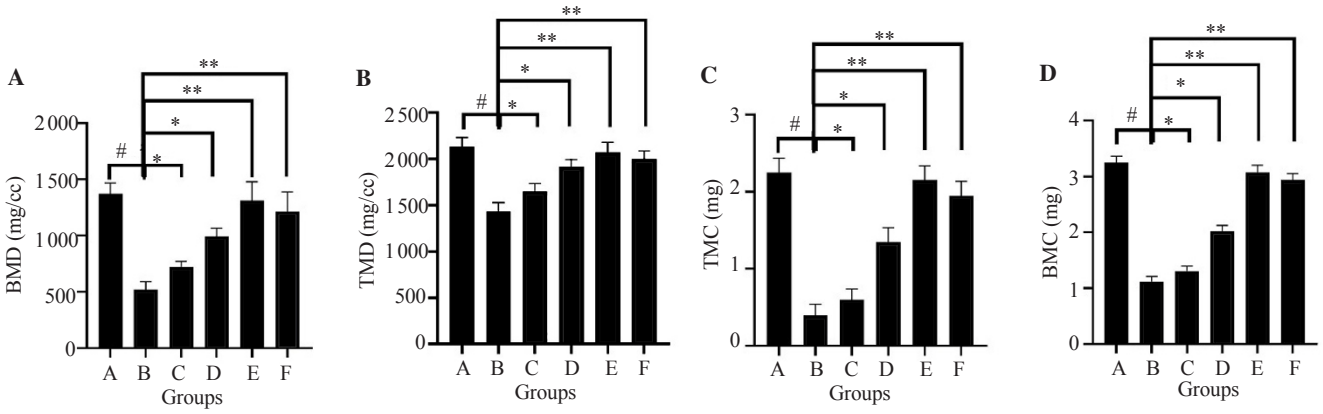


Figure 3. Effect of alpinumisoflavone on the bone parameters. A: bone mineral density (BMD), B: tissue mineralized density (TMD), C: tissue mineralized content (TMC), and D: bone mineral content (BMC). The whole data are shown as mean ± SEM. $^{\#}P$<0.001 as compared with normal control (Group A), $^{*}P$<0.05, $^{**}P$<0.001 as compared to GIOP group (Group B). Dunnett's *post hoc* test following ANOVA. Group A: Normal control, Group B: GIOP group, Group C: GIOP+alpinumisoflavone (5 mg/kg), Group D: GIOP+alpinumisoflavone (10 mg/kg), Group E: GIOP+alpinumisoflavone (15 mg/kg), Group F: GIOP+alendronate (2 mg/kg).

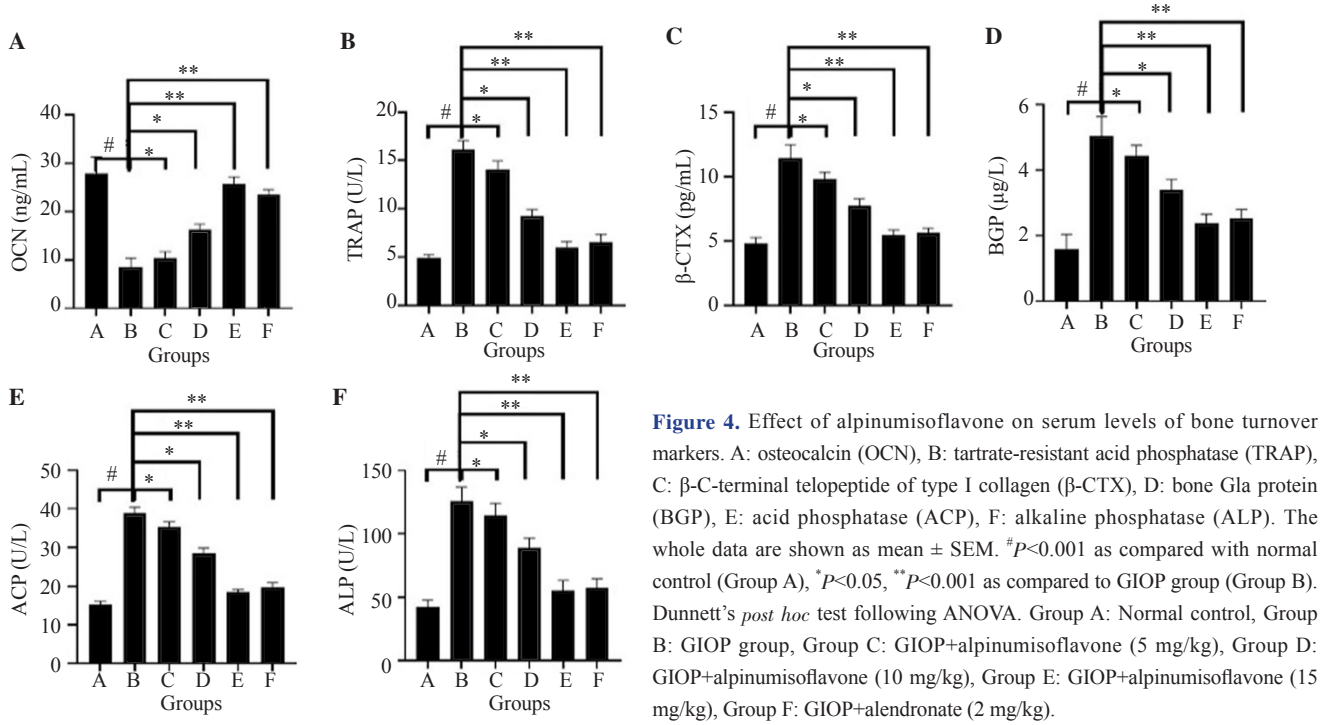


Figure 4. Effect of alpinumisoflavone on serum levels of bone turnover markers. A: osteocalcin (OCN), B: tartrate-resistant acid phosphatase (TRAP), C: β-C-terminal telopeptide of type I collagen (β-CTX), D: bone Gla protein (BGP), E: acid phosphatase (ACP), F: alkaline phosphatase (ALP). The whole data are shown as mean ± SEM. $^{\#}P$<0.001 as compared with normal control (Group A), $^{*}P$<0.05, $^{**}P$<0.001 as compared to GIOP group (Group B). Dunnett's *post hoc* test following ANOVA. Group A: Normal control, Group B: GIOP group, Group C: GIOP+alpinumisoflavone (5 mg/kg), Group D: GIOP+alpinumisoflavone (10 mg/kg), Group E: GIOP+alpinumisoflavone (15 mg/kg), Group F: GIOP+alendronate (2 mg/kg).

3.5. Oxidative stress parameters

GIOP group rats showed an augmented level of TBARS (Figure 5A) and suppressed level of GPx (Figure 5B), CAT (Figure 5C), GSH (Figure 5D), SOD (Figure 5E), GST (Figure 5F), and GR (Figure 5G). Alpinumisoflavone significantly (P<0.05) restored the level of oxidative stress parameters.

3.6. Cytokines

GIOP-induced osteoporosis rats showed increased levels of IL-1β (Figure 6A), IL-6 (Figure 6B), and TNF-α (Figure 6C), and decreased levels of TGF-β (Figure 6D) and IGF (Figure 6E). Alpinumisoflavone significantly (P<0.05) restored the level of cytokines.

GIOP group rats also exhibited the altered level of OPG (Figure 6F), RANKL (Figure 6G), and OPG/RANKL ratio (Figure 6H), while alpinumisoflavone significantly (P<0.05) restored the level of OPG, RANKL and OPG/RANKL ratio.

3.7. mRNA expression of apoptosis–related genes

GIOP group rats exhibited the enhancement of mRNA expression of *caspase–3* (Figure 7A), *caspase–6* (Figure 7B), *caspase–7* (Figure 7C), *caspase–9* (Figure 7D), *Bcl–2* (Figure 7E), *BclxL* (Figure 7F). Alpinimisoflavone remarkably suppressed the mRNA expression of caspase.

GIOP group rats also showed the boosted mRNA expression of *IAP1* (Figure 7G), *IAP2* (Figure 7H), and *XIAP* (Figure 7I), while alpinimisoflavone treatment remarkably suppressed the mRNA expression.

3.8. mRNA expression of bone morphogenetic proteins (BMPs) genes, ALP, OCN, osteopontin (OPN), and RunX2 genes

RT-qPCR results showed decreased mRNA expression of *BMP–2* (Figure 8A), *BMP–4* (Figure 8B), *BMP–6* (Figure 8C), *BMP–7* (Figure 8D), *BMP–9* (Figure 8E), *ALP* (Figure 8F), *OCN* (Figure 8G), *OPN* (Figure 8H), and *RunX2* (Figure 8I), while

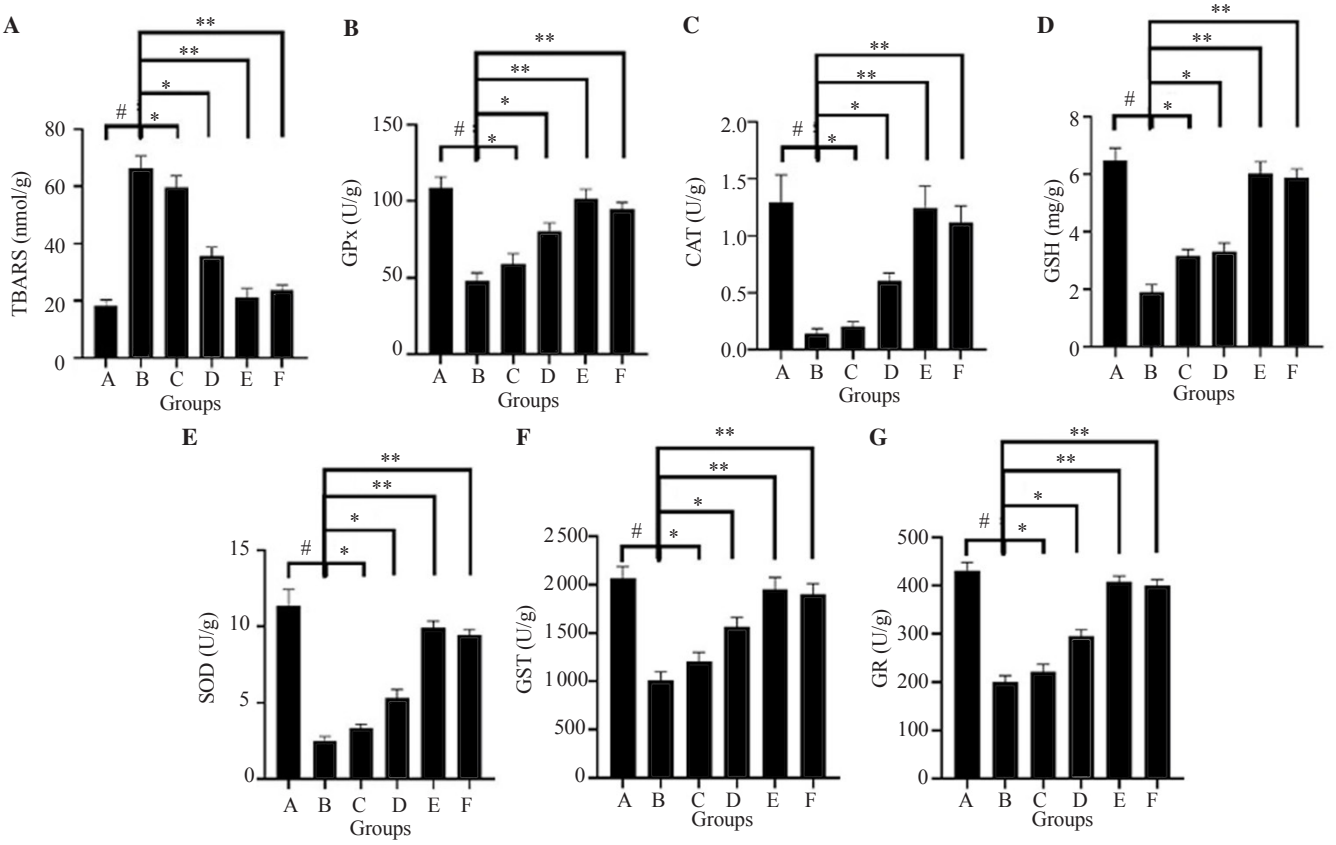


Figure 5. Effect of alpinumisoflavone on the oxidative stress parameters. A: thiobarbituric acid reactive substances (TBARS), B: glutathione peroxidase (GPx), C: catalase (CAT), D: glutathione (GSH), E: superoxide dismutase (SOD), F: glutathione-S-transferase (GST), and G: glutathione reductase (GR). The whole data are shown as mean ± SEM. $^{\#}P$<0.001 as compared with normal control (Group A), $^{*}P$<0.05, $^{**}P$<0.001 as compared to GIOP group (Group B). Dunnett's *post hoc* test following ANOVA. Group A: Normal control, Group B: GIOP group, Group C: GIOP+alpinumisoflavone (5 mg/kg), Group D: GIOP+alpinumisoflavone (10 mg/kg), Group E: GIOP+alpinumisoflavone (15 mg/kg), Group F: GIOP+alendronate (2 mg/kg).

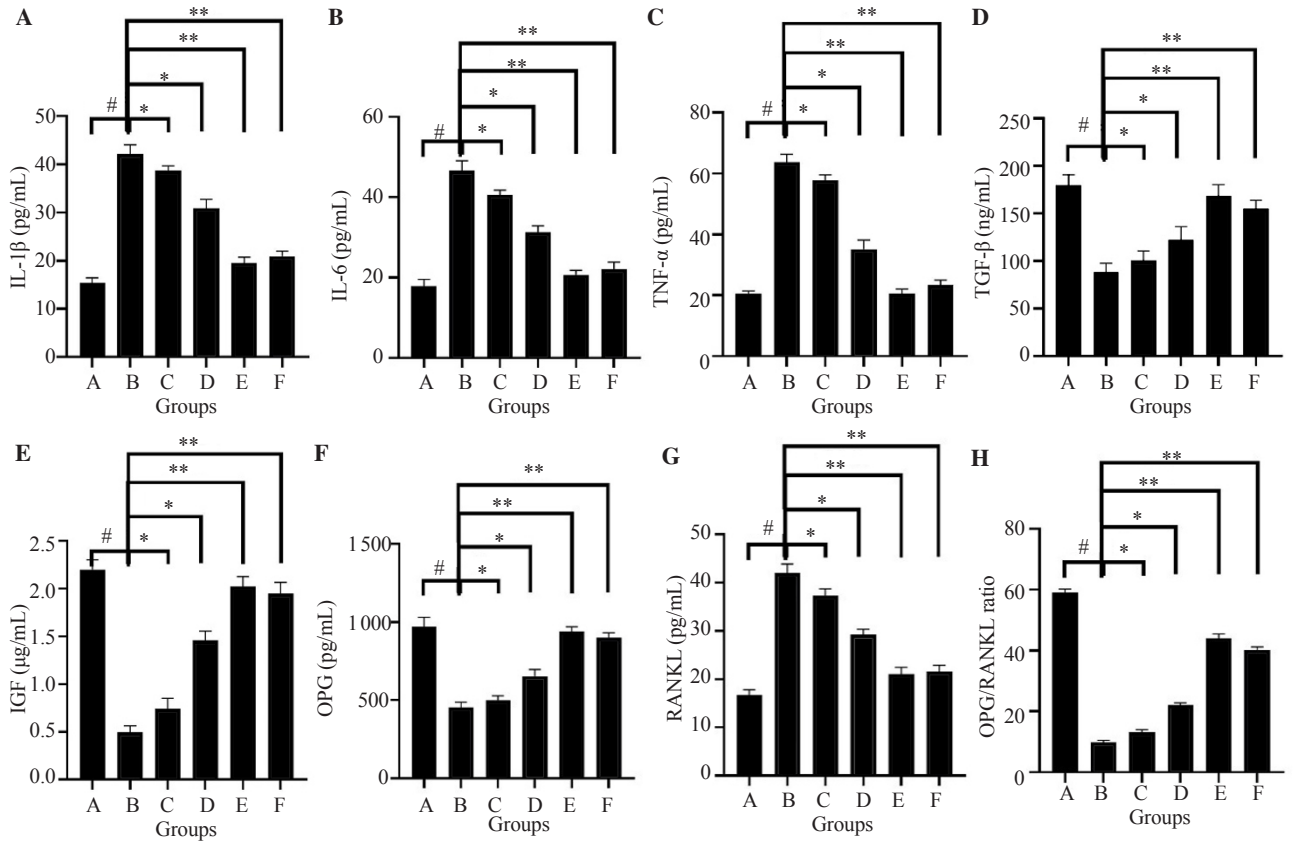


Figure 6. Effect of alpinumisoflavone on the cytokines. A: interleukin (IL)-β (IL-1β), B: IL-6, C: tumor necrosis factor (TNF)-α (TNF-α), D: TGF-β, E: insulin-like growth factor (IGF), F: OPG, G: RANKL, H: OPG/RANKL ratio. The whole data are shown as mean ± SEM. $^{\#}P<0.001$ as compared with normal control (Group A), $^{*}P<0.05$, $^{**}P<0.001$ as compared to GIOP group (Group B). Dunnett's *post hoc* test following ANOVA. Group A: Normal control, Group B: GIOP group, Group C: GIOP+alpinumisoflavone (5 mg/kg), Group D: GIOP+alpinumisoflavone (10 mg/kg), Group E: GIOP+alpinumisoflavone (15 mg/kg), Group F: GIOP+alendronate (2 mg/kg).

alpinimisoflavone treatment remarkably upregulated these mRNA expressions.

4. Discussion

Glucocorticoids impact bone metabolism through several mechanisms, including the inhibition of osteoblast function, osteoblast apoptosis induction, and osteoclast activity stimulation. This disruption in the bone formation and resorption balance can lead to reduced bone mineral density, compromised bone strength, and increased fracture risk[7,20]. DEX, a synthetic glucocorticoid medication, is commonly prescribed for its potent anti-inflammatory and immunosuppressive effects. However, long-term use of DEX or other glucocorticoids has been associated with adverse effects on bone health, including bone loss and an increased risk of developing osteoporosis. It is crucial to consider the potential bone-related side effects when using DEX or other glucocorticoids, especially in individuals requiring long-term treatment[7,21]. Proper monitoring and management of bone health, such as preventive measures, lifestyle modifications, and potential adjunctive therapies, may be necessary to mitigate the negative effects of glucocorticoid-induced bone loss[21,22].

Body weight indeed plays a crucial role in bone health and osteoporosis risk. Higher body weight typically correlates with increased bone mineral density and a decreased risk of osteoporosis. This is because body weight exerts mechanical stress on the bones during weight-bearing activities, stimulating bone remodeling and strengthening[23]. Conversely, low body weight, such as in cases of underweight or malnutrition, can lead to decreased bone mineral density and increased susceptibility to osteoporosis. The femur is the thigh bone and one of the major weight-bearing bones in the body. The weight or mass of the femur can be an indicator of bone health. In osteoporosis, there is a reduction in bone mass, including the femoral bone[24]. Lower femoral weight may suggest compromised bone density and increased fracture risk. The weights of the vagina and uterus are not direct indicators of bone health or osteoporosis risk. However, hormonal changes related to the reproductive system, such as estrogen deficiency during menopause, can contribute to bone loss and increased osteoporosis risk[25,26]. Estrogen is pivotal

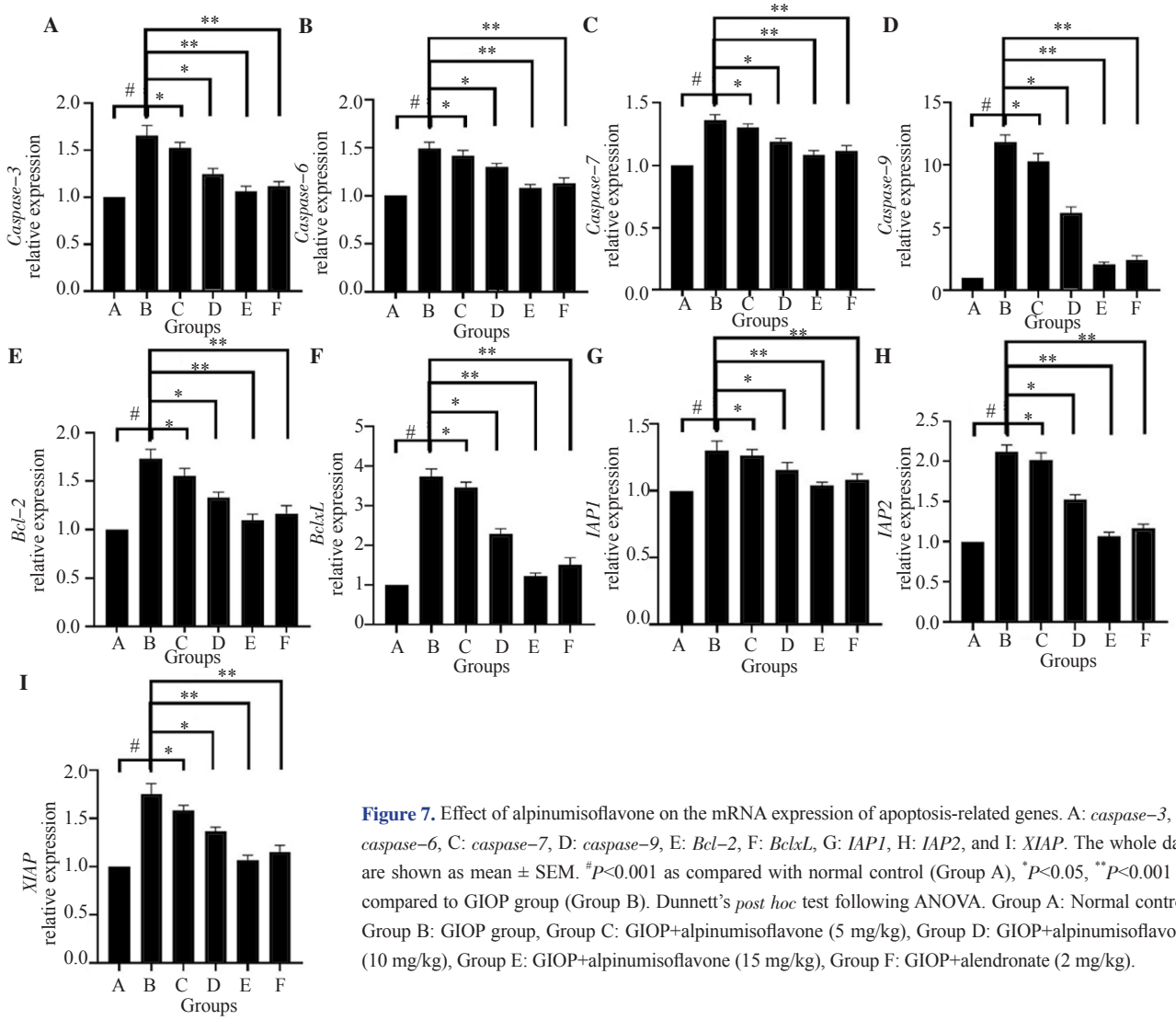


Figure 7. Effect of alpinumisoflavone on the mRNA expression of apoptosis-related genes. A: *caspase–3*, B: *caspase–6*, C: *caspase–7*, D: *caspase–9*, E: *Bcl–2*, F: *BclxL*, G: *IAP1*, H: *IAP2*, and I: *XIAP*. The whole data are shown as mean ± SEM. $^{\#}P<0.001$ as compared with normal control (Group A), $^{*}P<0.05$, $^{**}P<0.001$ as compared to GIOP group (Group B). Dunnett's *post hoc* test following ANOVA. Group A: Normal control, Group B: GIOP group, Group C: GIOP+alpinumisoflavone (5 mg/kg), Group D: GIOP+alpinumisoflavone (10 mg/kg), Group E: GIOP+alpinumisoflavone (15 mg/kg), Group F: GIOP+alendronate (2 mg/kg).

for preserving bone density, and its decline can hasten bone loss. Alterations in vaginal and uterine weights or conditions may indicate hormonal shifts linked to menopause, indirectly impacting bone health[27,28].

MicroCT analysis has a significant impact on studying and understanding osteoporosis. MicroCT, short for micro-computed tomography, is a non-invasive imaging method enabling high-resolution 3D visualization and quantification of bone microarchitecture[2]. MicroCT provides detailed information about trabecular and cortical bone parameters, including cortical thickness, trabecular thickness, trabecular separation, and number. These parameters are crucial for evaluating bone quality and detecting osteoporosis-related changes[2,29]. MicroCT can identify alterations in bone microarchitecture, such as trabecular thinning, increased trabecular separation, or cortical porosity, which are common in osteoporosis. MicroCT allows for measuring BMD, a key parameter in diagnosing and monitoring osteoporosis. BMD reflects the mineral content of bone and is closely related to bone strength and fracture risk[30]. MicroCT-based BMD measurements can be performed in both trabecular and cortical bone regions, providing a comprehensive assessment of bone density[2,30].

BMD measures the amount of minerals, such as calcium, present in a specific area or volume of bone. It is commonly measured using techniques like dual-energy X-ray absorptiometry (DXA). BMD is a key factor in diagnosing osteoporosis and assessing fracture risk. Low BMD indicates reduced bone mass and density associated with increased fracture susceptibility[31]. TMD represents the mineral density measurement in the entire body rather than specific bone regions. It provides an overall assessment of the body's mineral content, including bone mineral density. TMD measurements can be obtained using whole-body DXA scans or other imaging modalities. Low TMD may indicate a generalized decrease in bone mineral density and could indicate osteoporosis[32,33]. BMC refers to the total amount of minerals, such as calcium, in a specific bone or skeletal region. It is often measured using DXA or other imaging techniques. BMC provides information about the total mineral content of the bone, which contributes to its strength and resilience.

A lower BMC may indicate reduced bone mass and density,

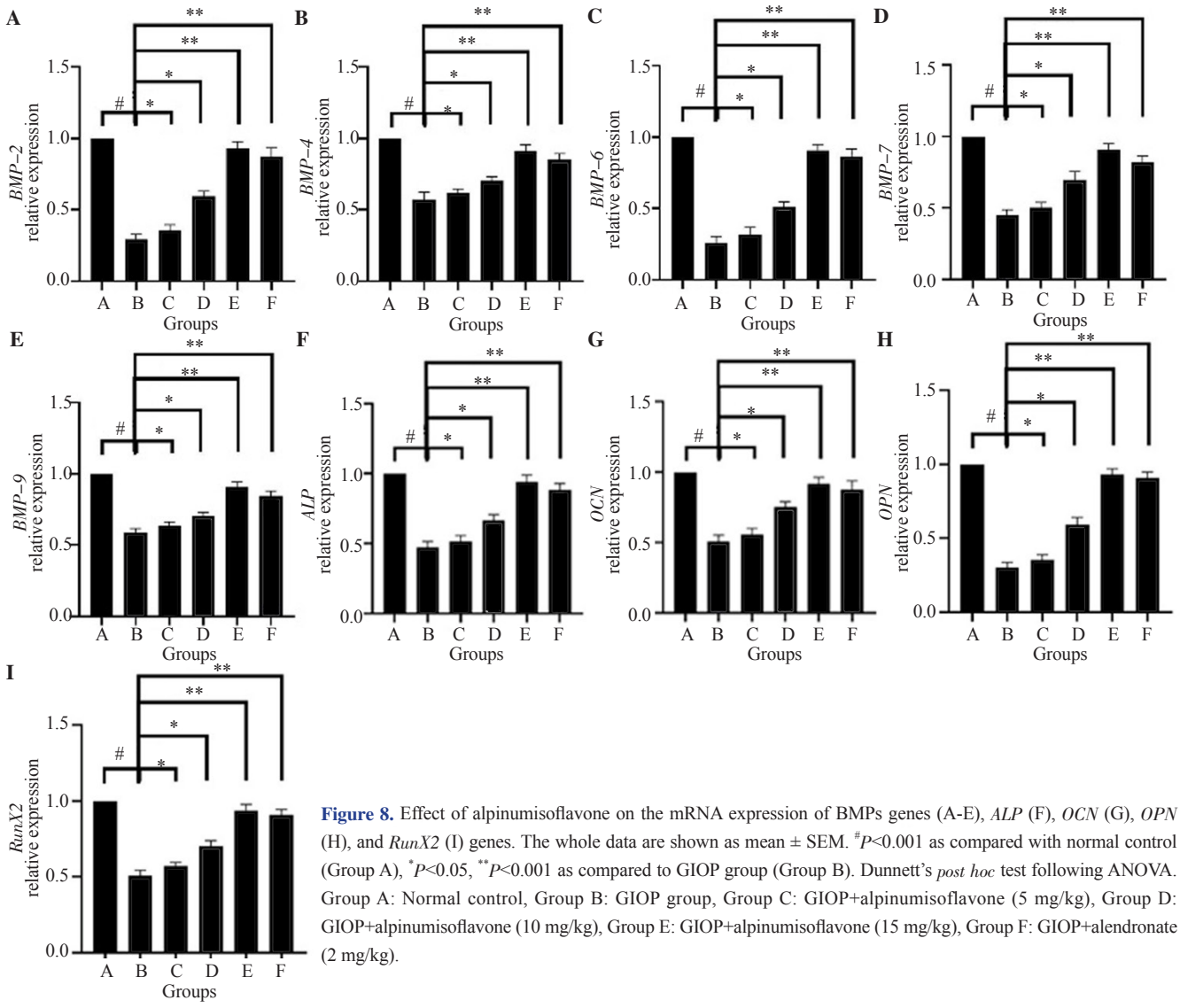


Figure 8. Effect of alpinumisoflavone on the mRNA expression of BMPs genes (A-E), *ALP* (F), *OCN* (G), *OPN* (H), and *RunX2* (I) genes. The whole data are shown as mean ± SEM. $^{\#}P<0.001$ as compared with normal control (Group A), $^{*}P<0.05$, $^{**}P<0.001$ as compared to GIOP group (Group B). Dunnett's *post hoc* test following ANOVA. Group A: Normal control, Group B: GIOP group, Group C: GIOP+alpinumisoflavone (5 mg/kg), Group D: GIOP+alpinumisoflavone (10 mg/kg), Group E: GIOP+alpinumisoflavone (15 mg/kg), Group F: GIOP+alendronate (2 mg/kg).

increasing the risk of fractures. TMC represents the total mineral content in the entire body, including all bones and non-skeletal tissues. It provides a comprehensive assessment of the body's mineral stores. TMC measurements can be obtained through whole-body DXA scans or other imaging methods. Monitoring TMC can help evaluate the body's overall mineral status, which is relevant to bone health.

OCN and BGP are both markers of bone formation. They are produced by osteoblasts and are involved in the mineralization and maturation of bone tissue. Measurement of OCN and BGP levels can provide insights into bone formation activity and the rate of bone turnover. The altered levels of OCN and BGP may indicate reduced bone formation and can be associated with osteoporosis. TRAP is an enzyme predominantly found in osteoclasts, the cells responsible for bone resorption. Elevated levels of TRAP can indicate increased osteoclast activity and bone resorption. Monitoring TRAP levels can help assess bone turnover and the degree of bone resorption associated with osteoporosis[2]. β-CTX is a marker of bone resorption, reflecting the osteoclasts' degradation of type I collagen, the primary constituent of bone matrix. Elevated levels of β-CTX indicate heightened bone resorption and can indicate osteoporosis. ACP and ALP are enzymes crucially involved in bone metabolism[34]. ACP is present in osteoclasts and is associated with bone resorption activity. ALP is produced by osteoblasts and is involved in bone formation. Changes in the levels of ACP and ALP can provide insights into alterations in bone turnover and the activity of bone cells in osteoporosis[2].

Oxidative stress arises from an imbalance between the production of ROS and the body's antioxidant defense mechanism. ROS, such as superoxide radicals and hydrogen peroxide, can cause damage to bone cells and disrupt the normal bone remodeling process. TBARS, GPx, CAT, GSH, and SOD are all biomarkers or enzymes associated with oxidative stress and antioxidant defense in the body[2,35]. TBARS are a group of compounds formed during lipid peroxidation, which occurs due to oxidative stress. Elevated levels of TBARS indicate increased oxidative damage to lipids in the body. GPx is an enzyme crucial for defending against oxidative damage. It functions by catalyzing the reduction of hydrogen peroxide (H_2O_2) and organic

hydroperoxides to less harmful molecules, thus helping to maintain cellular integrity and protect against oxidative stress. GPx relies on the antioxidant molecule GSH for its activity. CAT is another antioxidant enzyme that helps convert H_2O_2 into water and molecular oxygen. It acts as a defense mechanism against the harmful effects of H_2O_2, a ROS[25]. GSH is a potent intracellular antioxidant molecule that protects cells from oxidative damage. It acts as a reducing agent and can scavenge free radicals and ROS. GSH also contributes to regenerating other antioxidants like vitamins C and E.

Furthermore, GSH, as an important antioxidant, is involved in maintaining bone health. Decreased GSH levels have been reported in osteoporotic individuals, indicating a potential role in the pathogenesis of osteoporosis. SOD is an enzyme that catalyzes the conversion of superoxide radicals (O_2^-) into H_2O_2 and oxygen. SOD is the first line of defense against ROS and helps maintain the balance between oxidative stress and antioxidant defense[36]. Osteoporosis involves decreased bone mass and deterioration of bone tissue, elevating the risk of fractures. While the primary mechanism involves an imbalance between bone resorption and formation, oxidative stress is increasingly recognized as a contributing factor in the onset and progression of the disease. Studies have suggested that oxidative stress markers, including TBARS, GPx, CAT, GSH, and SOD, may be altered in individuals with osteoporosis. Increased levels of TBARS and decreased activities of antioxidant enzymes like GPx, CAT, and SOD have been observed in osteoporotic patients. This imbalance suggests that oxidative stress may contribute to the progression of osteoporosis by promoting bone loss and impairing bone cell function[11].

Cytokines indeed exert a notable influence on the development and progression of osteoporosis. These small proteins serve as signaling molecules within the immune system, orchestrating various cellular processes such as inflammation, bone remodeling, and the function of bone cells. IL-1β and TNF-α are pro-inflammatory cytokines that are involved in promoting bone resorption. They stimulate the activation of osteoclasts, leading to an imbalance between bone resorption and formation[25]. Increased levels of IL-1β and TNF-α have been observed in individuals with osteoporosis, contributing to accelerated bone loss. IL-6 exhibits a dual role in osteoporosis. In the short term, it can stimulate bone formation by enhancing the differentiation and activity of osteoblasts. However, IL-6 can contribute to bone loss in chronic inflammation by enhancing osteoclast formation and activity.

RANKL is a cytokine that promotes osteoclast formation and activation. It binds to its receptor, RANK, on osteoclast precursor cells, leading to their differentiation and bone resorption. Increased RANKL expression and activity have been associated with osteoporosis, contributing to excessive bone resorption. OPG, a cytokine, acts as a decoy receptor for RANKL, effectively preventing its binding to RANK. This interference inhibits osteoclast differentiation and activation, thereby regulating bone resorption and maintaining bone density[11,25]. OPG is a negative regulator of bone resorption and helps maintain bone homeostasis. Imbalances between RANKL and OPG, with increased RANKL levels and decreased OPG levels, favor excessive bone resorption and contribute to osteoporosis[25]. TGF-β is a multifunctional cytokine involved in various cellular processes, including bone remodeling[37]. TGF-β promotes the differentiation of osteoblasts and their synthesis of extracellular matrix proteins, leading to bone formation. However, chronic exposure to TGF-β can promote bone loss by stimulating bone resorption and inhibiting bone formation. These cytokines, along with other inflammatory factors, can disrupt the balance between bone resorption and formation, favoring excessive bone resorption and contributing to osteoporosis. Inflammatory conditions, such as rheumatoid arthritis or chronic glucocorticoid use, can further increase cytokine production and accelerate bone loss[37,38]. Understanding the role of cytokines in osteoporosis has led to the development of targeted therapies that aim to modulate the inflammatory response and restore bone homeostasis. For example, anti-resorptive medications, such as bisphosphonates or denosumab, can inhibit osteoclast activity by targeting RANKL, thereby reducing bone resorption[39,40]. Additionally, future research may uncover novel therapeutic strategies to modulate cytokine activity to prevent or treat osteoporosis.

It is well known that mesenchymal precursor cells derived from the bone-forming osteoblasts and the maturation of preosteoblasts differentiated from mesenchymal precursor cells play an important role in re-building of resorbed bone *via* elaborating a matrix that becomes mineralized[41,42]. These preosteoblasts become committed through signals that activate osteogenic genes. The active mature osteoblasts on the bone surface are distinguished by their morphological characteristics and temporal expression of various non-collagenous proteins, such as OPN and OCN, which are considered significant markers for mature osteoblasts[43].

BMPs play a crucial role in the differentiation and proliferation of osteoblastic cells and are potent stimulators of bone formation in various rodent models. BMP-2, in particular, is considered a significant marker and plays a pivotal role in enhancing the expression of osteogenic genes such as OCN and OPN, which are significant markers for mature osteoblasts. Furthermore, osteogenic BMP genes such as *BMP–2* and *BMP–7* have recently been approved for clinical applications in fracture healing, dental tissue engineering, and spinal fusion. Studies suggest that anabolic agents that enhance BMP expression or its signaling pathway could be utilized to treat osteoblast-related diseases, including bone regeneration and bone formation disorders[44,45]. Reduction in the expression of BMP-2 and BMP-4 indicates dysfunction in osteogenesis. The molecular switches and network of activities for bone expansion and osteoblastic differentiation involve BMP-induced transcription factors such as RUNX2, which acts as the master transcription factor for osteogenesis and is activated by the BMP-2 signaling pathway[43]. GIOP-induced osteoporosis rodents exhibited the alteration in the mRNA expression of BMPs, and

alpinumisoflavone restored the mRNA expression and suggested the bone protective effect.

Alpinumisoflavone treatment significantly improved body weight and the weight of the vagina and uterus in the rat model. The treatment also restored bone parameters, oxidative stress parameters, cytokines, and inflammatory markers. Alpinumisoflavone demonstrated potential in bone metabolism by reducing bone resorption and enhancing bone formation. It significantly suppressed the activation of caspase-3 and caspase-9 and also upregulated the expression of osteogenic gene markers such as *BMPs*, *OPN*, *ALP*, *RUNX2*, and *OCN*. These results suggest that alpinumisoflavone may be a promising candidate for preventing or treating GIOP. Further studies are needed to confirm these findings and evaluate the long-term efficacy and safety of alpinumisoflavone in different models and potentially in clinical settings.

The limitation of the study focused solely on GIOP. Other models of osteoporosis, such as postmenopausal or age-related osteoporosis, were not evaluated, limiting the generalizability of the findings. The study measured biochemical and molecular markers, but did not assess functional outcomes such as bone strength, fracture rates, or mobility, which are clinically relevant in osteoporosis management. These limitations highlight the need for further research to confirm the bone-protective effects of alpinumisoflavone and to address these gaps for a more comprehensive understanding of its potential in treating osteoporosis.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

JL performed the experimental study. JLQ and GWZ interpreted the experimental data. XBJ designed the experimental study. All the authors equally contributed to proof reading.

References

[1] Ensrud KE, Crandall CJ. Osteoporosis. *Ann Intern Med* 2024; **177**(1): ITC1-ITC16.

[2] Wang H, Yang L, Chao J. Antiosteoporosis and bone protective effect of dieckol against glucocorticoid-induced osteoporosis in rats. *Front Endocrinol (Lausanne)* 2022; **13**: 932488.

[3] Chakrabarti K, Joseph McCune W. Glucocorticoid-induced osteoporosis in premenopausal women: Management for the rheumatologist. *Curr Opin Rheumatol* 2023; **35**(3): 161-169.

[4] Laurent MR, Goemaere S, Verroken C, Bergmann P, Body JJ, Bruyère O. Prevention and treatment of glucocorticoid-induced osteoporosis in adults: Consensus recommendations from the belgian bone club. *Front Endocrinol* 2022; **13**: 908727.

[5] Liang H, Zhao J, Tian T. Pharmacological interventions for glucocorticoid-induced osteoporosis: An umbrella review. *Horm Metab Res* 2023; **55**(8): 511-519.

[6] Zhang L, Li X, Ying T, Wang T, Fu F. The use of herbal medicines for the prevention of glucocorticoid-induced osteoporosis. *Front Endocrinol* 2021; **12**: 744647.

[7] Adami G, Saag KG. Glucocorticoid-induced osteoporosis update. *Curr Opin Rheumatol* 2019; **31**: 388-393.

[8] Lane NE. Glucocorticoid-induced osteoporosis: New insights into the pathophysiology and treatments. *Curr Osteoporos Rep* 2019; **17**(1): 1-7.

[9] Castrén E, Kojima M. Brain-derived neurotrophic factor in mood disorders and antidepressant treatments. *Neurobiol Dis* 2017; **97**: 119-126.

[10] Alattar R, Abdel Alim AA, Abdelmetal S, Abdel Aziz S. Corticosteroid-induced osteoporosis and osteonecrosis: The role of oxidative stress. *Zagazig Vet J* 2020; **48**(1): 1-11.

[11] Kamaruzzaman M, Thanu A, Yusof M, Soelaiman I, Ramli E. Kelulut honey ameliorates glucocorticoid induced osteoporosis *via* its antioxidant activity in rats. *Asian Pac J Trop Biomed* 2019; **9**(12): 493-500.

[12] Xia C, Dong X, Li H, Cao M, Sun D, He S. Cancer statistics in China and United States, 2022: Profiles, trends, and determinants. *Chin Med J (Engl)* 2022; **135**(5): 584-590.

[13] Sun X, Gan L, Li N, Sun S, Li N. Tabersonine ameliorates osteoblast apoptosis in rats with dexamethasone-induced osteoporosis by regulating the Nrf2/ROS/Bax signalling pathway. *AMB Express* 2020; **10**: 165.

[14] Ma Q. Role of Nrf2 in oxidative stress and toxicity. *Ann Rev Pharmacol Toxicol* 2013; **53**: 401-426.

[15] Shi XM, Chutkan N, Hamrick MarkW, Isales CM. Mechanism of glucocorticoid-induced osteoporosis: An update. In: Qian XX (ed.) *Glucocorticoids – New recognition of our familiar friend*. IntechOpen; 2012, p. 7-16.

[16] Komori T. Glucocorticoid signaling and bone biology. *Horm Metab Res* 2016; **48**(11): 755-763.

[17] Hachemi Y, Rapp AE, Picke AK, Weidinger G, Ignatius A, Tuckermann J. Molecular mechanisms of glucocorticoids on skeleton and bone regeneration after fracture. *J Mol Endocrinol* 2018; **61**: R75-R90.

[18] Reeves PG, Nielsen FH, Fahey GC. AIN-93 purified diets for laboratory

rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 1993; **123**(11): 1939-1951.

[19]Tolba MF, El-Serafi AT, Omar HA. Caffeic acid phenethyl ester protects against glucocorticoid-induced osteoporosis *in vivo*: Impact on oxidative stress and RANKL/OPG signals. *Toxicol Appl Pharmacol* 2017; **324**: 26-35.

[20]Xavier A, Toumi H, Lespessailles E. Animal model for glucocorticoid induced osteoporosis: A systematic review from 2011 to 2021. *Int J Mol Sci* 2022; **23**(1): 377.

[21]Cho SK, Sung YK. Update on glucocorticoid induced osteoporosis. *Endocrinol Metab* 2021; **36**: 536-543.

[22]Compston J. Glucocorticoid-induced osteoporosis: An update. *Endocrine* 2018; **61**: 7-16.

[23]Feng M, Zhang R, Gong F, Yang P, Fan L, Ni J. Protective effects of necrostatin-1 on glucocorticoid-induced osteoporosis in rats. *J Steroid Biochem Mol Biol* 2014; **144**(PB): 455-462.

[24]Coin A, Sergi G, Benincà P, Lupoli L, Cinti G, Ferrara L. Bone mineral density and body composition in underweight and normal elderly subjects. *Osteoporos Int* 2000; **11**(12): 1043-1050.

[25]Mo H, Zhang N, Li H, Li F, Pu R. Beneficial effects of *Cuscuta chinensis* extract on glucocorticoid-induced osteoporosis through modulation of RANKL/OPG signals. *Brazilian J Med Biol Res* 2019; **52**(12): e8754.

[26]Zhang ZF, Min JK, Wang D, Zhong JM. Pinoresinol diglucoside exhibits protective effect on dexamethasone-induced osteoporosis in rats. *Trop J Pharm Res* 2016; **15**(11): 2451-2457.

[27]Gu G, Hentunen TA, Nars M, Härkönen PL, Väänänen HK. Estrogen protects primary osteocytes against glucocorticoid-induced apoptosis. *Apoptosis* 2005; **10**(3): 583-595.

[28]Utaminingtyas NI, Ma'arif B, Megawati DS, Atmaja RRD. Activity of 70% ethanol extract of *Chrysophyllum cainito* in increasing vertebrae trabecular bone density in female mice. *Maj Obat Tradis* 2018; **23**(3): 119.

[29]Kim Y, Brodt MD, Tang SY, Silva MJ. MicroCT for scanning and analysis of mouse bones. In: Walker JM (ed.) *Methods in molecular biology*. Springer; 2021, p. 169-198.

[30]Qiu Y, Tang C, Serrano-Sosa M, Hu J, Zhu J, Tang G. Bone microarchitectural parameters can detect oxytocin induced changes prior to bone density on mitigating bone deterioration in rabbit osteoporosis model using micro-CT. *BMC Musculoskelet Disord* 2019; **20**(1): 560.

[31]Taylor AD, Saag KG. Anabolics in the management of glucocorticoid-induced osteoporosis: An evidence-based review of long-term safety, efficacy and place in therapy. *Core Evid* 2019; **14**: 41-50.

[32]Hozayen WG, El-Desouky MA, Soliman HA, Ahmed RR, Khaliefa AK. Antiosteoporotic effect of *Petroselinum crispum*, *Ocimum basilicum* and *Cichorium intybus* L. in glucocorticoid-induced osteoporosis in rats. *BMC Complement Altern Med* 2016; **16**(1): 165.

[33]Zhao J, Li Y, Zhang H, Shi D, Li Q, Meng Y. Preventative effects of metformin on glucocorticoid-induced osteoporosis in rats. *J Bone Miner Metab* 2019; **37**(5): 805-814.

[34]Calvo MS, Eyre DR, Gundberg CM. Molecular basis and clinical application of biological markers of bone turnover. *Endocr Rev* 1996; **17**: 333-368.

[35]Yang L, Zhang B, Liu J, Dong Y, Li Y, Li N, et al. Protective effect of acteoside on ovariectomy-induced bone loss in mice. *Int J Mol Sci* 2019; **20**(12): 2974.

[36]Cho CW, Park YS, Yan XT, Kim HJ, Kim YH, Kim KT. Quantitation of phenolic compounds related to antioxidant and antiosteoporosis activities in ripe and unripe maesil (*Prunus mume*). *J Food Qual* 2020; **2020**: 1-13.

[37]Hirao T, Kim BG, Habuchi H, Kawaguchi K, Nakahari T, Marunaka Y. Transforming growth factor-β1 and bone morphogenetic protein-2 inhibit differentiation into mature ependymal multiciliated cells. *Biol Pharm Bull* 2023; **46**(1): 111-122.

[38]Si X, Liu X, Li J, Wu X. Transforming growth factor-β1 promotes homing of bone marrow mesenchymal stem cells in renal ischemia-reperfusion injury. *Int J Clin Exp Pathol* 2015; **8**(10): 12368-12378.

[39]Zhao Y, Wang HL, Li TT, Yang F, Tzeng CM. Baicalin ameliorates dexamethasone-induced osteoporosis by regulation of the RANK/RANKL/OPG signaling pathway. *Drug Des Devel Ther* 2020; **14**: 195-206.

[40]Ni X, Wu B, Li S, Zhu W, Xu Z, Zhang G. Equol exerts a protective effect on postmenopausal osteoporosis by upregulating OPG/RANKL pathway. *Phytomedicine* 2023; **108**: 154509.

[41]Florencio-Silva R, Sasso GRDS, Sasso-Cerri E, Simões MJ, Cerri PS. Biology of bone tissue: Structure, function, and factors that influence bone cells. *BioMed Res Int* 2015; **2015**: 421746.

[42]Grabowski P. Physiology of bone. *Endocr Dev* 2015; **28**(6): 33-55.

[43]Kim J, Lee H, Kang KS, Chun KH, Hwang GS. Protective effect of Korean Red Ginseng against glucocorticoid-induced osteoporosis *in vitro* and *in vivo*. *J Ginseng Res* 2015; **39**(1): 46-53.

[44]Deng ZH, Li YS, Gao X, Lei GH, Huard J. Bone morphogenetic proteins for articular cartilage regeneration. *Osteoarthr Cartil* 2018; **26**: 1153-1161.

[45]Tang H, Zhang X, Xue G, Xu F, Wang Q, Yang P. The biology of bone morphogenetic protein signaling pathway in cerebrovascular system. *Chin Neurosurg J* 2021; **7**(1): 36.

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Antiosteoporosis and bone protective effect of alpinumisoflavone in steroid-induced osteoporosis rats *via* alteration of apoptosis, inflammatory and RANK/RANKL/OPG signaling pathway

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Supplementary Table 1. List of primer sequences.

S. No	Gene	Primers	
		Reverse (5'- 3')	**Forward (5'- 3')**
1	*ALP*	CTGGGCCTGGTAGTTGTTGT	GCTGATCATTCCCACGTTTT
2	*OPN*	TGGCATCAGGATACTGTTCATC	CGATGATGATGACGATGGAG
3	*RunX2*	GGACCGTCCACTGTCACTTT	GCCGGGAATGATGAGAACTA
4	*OCN*	TTTGTAGGCGGTCTTCAAGC	AAGCAGGAGGGCAATAAGGT
5	*BMP-2*	TCAACTCAAATTCGCTGAGGA	GGGACCCGCTGTCTTCTAGT
6	*BMP-4*	AGCCGGTAAAGATCCCTCAT	CCTGGTAACCGAATGCTGAT
7	*BMP-6*	ACTCTTGCGGTTCAAGGAGTG	CAACGCCCTGTCCAATGAC
8	*BMP-7*	AAGGTCTCGTTGTCAAATCGC	CGATACCACCATCGGGAGTTC
9	*BMP-9*	CCTTTGTGGGGAACTTGAGA	CAGGTGAGAGCCAAGAGGAG
10	*Bcl2*	CTGTTGACGCTCTCCACACA	ACTTCTCTCGTCGCTACCGT
11	*Bcl-Xl*	TCAGGAACCAGCGGTTGAAG	CGTAGACAAGGAGATGCAGG
12	*IAP-1*	GGACCATTAGTCTTGTTCAG	AAGTGTGTATGGACCGAGAG
13	*XIAP*	GGTTGAACGTAATGACGGTG	ATACGGAGGATGAGTCAAGT
14	*IAP-2*	CAGCTTCTGATGTCCAACAA	TGTGTATGGACAGAGAGGTT
15	*caspase-3*	AGTTTCGGCTTTCCAGTCAGAC	AGCAGCTTTGTGTGTGTGATTCTAA
16	*caspase-6*	CCAGGAGCCATTCACAGTTTCT	AGACAAGCTGGACAACGTGACC
17	*caspase-7*	TAAGGCGCTGGTGGATATGG	GGAGGACTATGGTGATTGGAGC
18	*caspase-9*	GGTCTAGATTATGATGTTTTAAAGAAAAG	GGATCCATGCCCAGACCAGTGGACATTGG
19	*β-actin*	TCCTTAATGTCACGCACCATTT	TCACCCACACTCTGCCCAT