

Exploring the Beneficial Role of Ononin in Preventing Ovariectomy-Induced Osteoporosis In Rats Via Osx And Runx2 Pathway

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ABSTRACT

Background: Osteoporosis is a multifactorial bone disease that progresses without notice until attaining a chronic stage. It causes socio-economic burden, higher mortality rate, and increased bone fractures. Ononin, an isoflavone present in numerous herbal Chinese medicinal formulations such as *Astragali radix* and red clover, has been reported to have potent therapeutic properties.

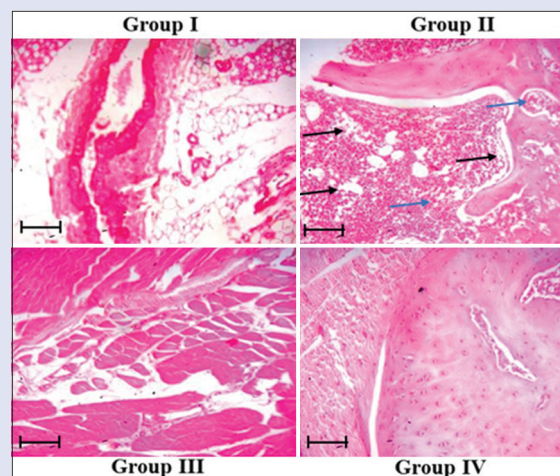
Objectives: In this study, we aimed to evaluate the efficacy of ononin against ovariectomy(OVX)-induced osteoporosis in female rats. **Materials and Methods:** Ovaries were removed surgically to develop the animal model of OVX. Animals ($n = 4$) were grouped into control, OVX induced, OVX rats treated with ononin (10 mg/kg body weight), and OVX rats treated with 20 mg/kg body weight of ononin.

Results: OVX rats treated with ononin showed improved body weight, uterus index, inflammatory and bone markers, serum lipid profile, and calcium level. Ononin downregulated the expression of estrogen (E2), acid phosphatase, and bone gamma-carboxyglutamate (Gla) protein in OVX rats. It also restored trabecular area and thickness, which was evidenced by histomorphometrical and histopathological examination. Ononin attenuated the expression of alkaline phosphatase, osterix, and Runx2 in OVX rats. **Conclusion:** In summary, our results suggest that ononin could be used as an ideal therapeutic agent to prevent and treat bone-associated disorders.

Key words: Femur, flavonoids, ononin, osteoporosis, ovariectomy

SUMMARY

- Ononin was found to alleviate osteoporosis in OVX rats by increasing the body weight, restoring the levels of lipid, and maintaining a healthy uterus index.
- The efficacy of ononin in preventing osteoporosis among the other flavonoid compounds was found to be remarkable and can be considered as a potent therapeutic agent for treating bone disorders.



Abbreviations used: OVX: Ovariectomy-induced; ACP: Phosphatase; BGP: Bone gla protein, HDLC: high-density lipoprotein cholesterol; LDLC: Low-density lipoprotein cholesterol; TC: Total cholesterol; TG: Triglycerides; E2: Estrogen; and ALP: Alkaline phosphatase.

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INTRODUCTION

Osteoporosis is a metabolic disorder and a multifactorial bone disease that progresses without notice until attaining the chronic stage and ends up with intricate pathophysiology.^[1,2] It is caused due to an imbalance between the formation of osteoblasts and the resorption of osteoclasts. It is mainly characterized by a reduced bone mass and density that eventually results in increased fragility and fracture of bones.^[3-5] The initiation of osteoporosis can be detected by a reduction in the levels of estrogen (E2) serves as an index to assess the initiation of reduction in bone density, which can cause osteoporosis.^[6] Osteoporosis is emerging as a major problem of concern among the elderly and postmenopausal women, which is increasing at an alarming rate.^[7] Patients with osteoporosis suffer from weakness which disturbs their day-to-day activities and quality of life. The mortality rate of

osteoporosis is high, which is associated with a lack of nutrition, disorders of the endocrine system, and hereditary issues.^[7,8] Worldwide, a significant increase in the ageing population and reduced quality of life has resulted in a high risk of developing osteoporosis.^[9]

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Osteoporosis causes an increased socio-economic burden, a higher mortality rate, and an increased bone fractures.^[10] However, literature is scarce regarding the role of pathological events in the development of osteoporosis. Various pharmacological drugs have been currently used in the treatment of osteoporosis, but they show undesirable side effects in humans.^[11] Most of the drugs are still under investigation, and pharmacological treatments such as hormone therapy (HT) and selective estrogen receptor modulators (SERMs) have been shown to be beneficial against osteoporosis.^[12] Patients with osteoporosis undergoing long-term treatment with HT have reported toxic side effects, which predispose them to increased risk of ovarian, endometrial, and breast cancers.^[13] However, all the treatment strategies for HT possess side effects such as gastrointestinal discomfort and malignancies.^[14,15] Drugs used to treat osteoporosis such as corticosteroids, immune suppressors, and 5-aminosalicylates are potent SERMs, but they cause various side effects such as hypertension, disorders of the kidney, diarrhea, and fever.^[16] Women have to undergo ovariectomy (OVX) procedures to maintain a balance between bone formation and resorption processes. However, the chemotherapeutics treatment for osteoporosis causes various adverse side effects such as pain in the muscles, nausea, heartburn, and inability to swallow.^[17]

Osteoporosis can be prevented by maintaining normal levels of vitamin D and calcium. Regular physical activity and avoiding smoke and reducing the intake of caffeine and alcohol might prevent the formation of osteoporosis.^[18] Diet rich in calcium and vitamin D would help in increasing bone density and preventing osteoporosis.^[19-21] Elderly and postmenopausal women suffer from bone loss caused due to increased levels of oxidative stress and decreased levels of the antioxidant defence system. Oxidative stress promotes osteoclastic function thereby resulting in bone loss.^[22-24]

Therefore, treatment strategies for osteoporosis should include regular intake of healthy food in addition to increased physical activity.^[25,26] However, additional studies are highly warranted to explore alternative ways that could diminish the bone loss resulting from osteoporosis. Nonhormonal methods of treatment or compounds obtained from natural substances might be highly beneficial in preventing osteoporosis.^[27]

Flavonoids are natural free radical scavengers. Isoflavonoids such as ononin, formononetin, calycosin, and their related glycosides inhibit glucosidase activity, maintain the metabolism of fat and glucose, and decrease the risk of developing cardiovascular diseases.^[28] These substances can enhance energy production, boost up the immune system, and help in the rejuvenation of the skin.^[29] Both ononin and formononetin isoflavones in numerous herbal Chinese medicinal formulations such as *Astragali radix* and red clover. Formononetin and ononin has been proven to have antineoplastic,^[30,31] antimicrobial,^[32] antioxidant,^[33,34] antiarthritic,^[35] antiinflammatory,^[36,37] vasorelaxant,^[38] cardioprotective,^[39] hypolipidemic,^[40] antitumor,^[41] and neuroprotective^[42] activity. Ononin is a glycosylated derivative of formononetin which is reported to have potent antiinflammatory activity against lipopolysaccharide-induced inflammation.^[43] It shows antiangiogenic activity and acts as a tumor modulator.^[44,45] With this information, in this study, we aimed to assess the efficacy of ononin and its mechanism of action in preventing osteoporosis in ovariectomized rats.

MATERIALS AND METHODS

Chemicals and reagents

Ononin (≥ 99.0), ketamine, and other chemicals were purchased from the Sigma Aldrich, USA. Other reagents and solvents used were of analytical grade.

Experimental design

Female Sprague rats (7–9 weeks old and weighing approximately 200–250 g) were used in this study. The animals were maintained under standard laboratory conditions in an animal house in an air-conditioned environment. Animals were acclimatized for 7 days in the laboratory before conducting the experiments. Throughout the experimental period, animals were provided with a standard pellet diet and free access to water. All the experiments performed in this study were approved and conducted according to standard guidelines of the Institutional animal ethical committee (2020–142). The animals were categorized into four groups of six animals each. Group, I rats are sham-operated control rats (SHAM), group II are ovariectomized (OVX) rats, group III are OVX rats treated with 10 mg/kg body weight of ononin/day orally for 16 weeks, and group IV are OVX rats treated with 20 mg/kg body weight of ononin/day for 16 successive weeks.

The surgical procedure was conducted following ketamine anesthesia to rats. Immediately after the animals were anesthetized, dorsolateral incisions were made with the help of scissors. The skin of the rats and the muscles of the dorsal region was cut and the fat tissues surrounding the ovaries were excised. Ovaries were removed by making a cut, and the region between the uterine horn and the fallopian tubes was clamped. Immediately after the removal of the ovaries, the skin that was cut was closed by suture. OVX was performed based on a previous report.^[45] In SHAM-operated rats, the procedure was conducted and the ovaries were left exposed.

Biochemical marker analysis

Blood samples were collected in tubes from the femoral artery and allowed to clot. Then, serum was separated via centrifugation at 1,200 g for 10 min, which was used to analyze the biochemical markers such as phosphorus, calcium, acid phosphatase (ACP), bone Gla protein (BGP), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), triglycerides (TG), E2, and alkaline phosphatase (ALP) according to the protocol given by the manufacturer in commercially available ELISA kit (BioCompare, USA). In addition to this, serum levels of tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-1 β was also analyzed according.^[46,47]

Determination of organ weight

The left femur, uterus, and vagina were removed and the weight was measured before and after drying to foresee their weight. Organ weight and their equivalent organ coefficients were calculated based on the procedure reported earlier.^[48,49] They were calculated using the internal organ weight and the equivalent body weight of respective animals.

Biomechanical properties and bone mineral density (BMD) were tested by extracting the left femur of rats and examining them using instructions as described.^[48,49]

Analysis of bone turnover markers in serum

Serum levels of calcium, creatinine, phosphorus, and tartrate-resistant acid phosphatase (TRAP) were analyzed using commercially available ELISA kits (Thermo Fisher, USA) and the values were read using a double beam spectrophotometer. Serum samples of animals were examined for the dimensions of Receptor activator of nuclear factor κ B (RANK) and its ligand (RANKL) and Osteoprotegerin (OPG)^[50] using commercially procured ELISA kits (Abcam, USA).

Real-time polymerase chain reaction (RT-PCR) analysis

Femur bones extracted from experimental animals were dissolved and crushed using liquid nitrogen and further mixed with a ribonucleic

acid (RNA) reagent and the RNA was extracted according to the standard procedures.^[51] The extracted RNA was quantified and detected using agarose gel electrophoresis. RT-PCR was performed and the mRNA expression of Runx2, Osx, and ALP was determined and normalized to that of the house-keeping gene (β -actin). The following conditions were set for PCR analysis: initial denaturation for 15 sec at 95°C, annealing at 64°C followed by primer extension for 30 sec at 72°C for about 40–45 cycles.

Histomorphometrical and histopathological examination of bone

Femur bone sections were stored in formalin and embedded in paraffin wax. The sections were then cut using a microtome and stained using hematoxylin and eosin (H&E) for both histopathological and histomorphometrical analysis. The H and E staining was performed according to the previously described protocol.^[52] Histomorphometric analysis was performed using image analyzing software as mentioned earlier.^[53]

Statistical analysis

In this study, results are presented as mean $\pm$ standard deviation. Data were compared with a one-way analysis of variance. Intergroup comparisons were made using the least significant difference method. Statistical Package for Social Sciences software (SPSS Inc., IBM, USA) was used for the processing of data and the significance level was given as $P < 0.05$.

RESULTS

Efficacy of ononin in body weight, uterine weight, and vaginal weight and uterus index of OVX rats

Animals in all experimental groups showed a marked increase in body weight throughout the experimental period [Figure 1]. The body weight of control animals was found to increase between 4 and 8 weeks when compared with treated animals. Even though body weight was found to be lower in the treatment group than that of the control

group, the body mass of all experimental animals was found to increase significantly ($P < 0.05$). Treatment with ononin showed a marked decrease in weight gain in OVX rats. The uterine index was found to be higher in control animals [Figure 1]. Administration of ononin (10 and 20 mg/kg of body weight) to groups and group IV animals displayed upregulated uterine index in rats that were ovariectomized. Uterine weight was found to be higher in control animals [Figure 1]. Ononin (10 and 20 mg/kg of body weight) in groups III and IV animals caused an increase in uterine weight in OVX rats. Vaginal weight was also found to be lower in OVX-induced rats [Figure 1]. Ononin (10 and 20 mg/kg of body weight) in groups III and IV animals increased the weight of the uterus in ovariectomized rats.

Efficacy of ononin in femoral wet and dry weights and organ coefficient of OVX rats

Femoral wet and dry weights and their respective organ coefficients were evaluated in all experimental groups [Figure 2]. Femoral wet and dry weights were found to be reduced in ovariectomized rats. The organ coefficient of the femur was also found to be lowered in ovariectomized rats (Group II). The femoral weight and organ coefficient were markedly reduced in groups III and IV animals. Significant changes were observed in OVX rats and Group III with respect to their uterine mass, organ coefficient, and femoral weight of OVX rats, which demonstrates the efficacy of ononin in OVX rats.

Efficacy of ononin in femoral fitness and biomechanical properties of OVX rats

Bone fitness of the OVX rats was examined for the efficacy of ononin on the biomechanical properties and femoral bone length [Figure 3]. The femoral bone length was decreased in OVX rats than that of control animals. Group III showed improvement in femoral length than that of group II animals. However, the femoral bone length of groups III and IV animals were found to be less than that of the control animals. Femoral bone mineral density was reduced in OVX rats than that of control animals. Ononin-treated OVX rats showed improvement in bone mineral density than that of group II animals. The eminence of bone was

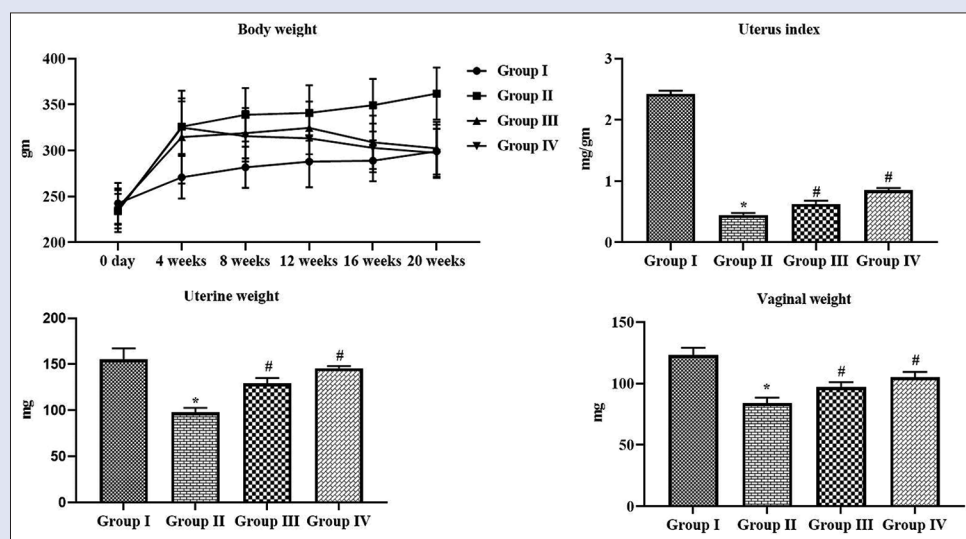


Figure 1: Efficacy of ononin in body, uterine and vaginal weight, and in uterus index of OVX rats. Group I—sham (SHAM) operated control rats, group II—ovariectomized (OVX) rats, group III—OVX rats treated with 10 mg/kg body weight of ononin, and group IV—OVX rats treated with 20 mg/kg body weight of ononin. Data was represented as the mean $\pm$ standard deviation (SD) of triplicates ($n = 6$). * $P < 0.05$ —control compared with other groups and ** $P < 0.05$ —OVX-induced group compared with other groups

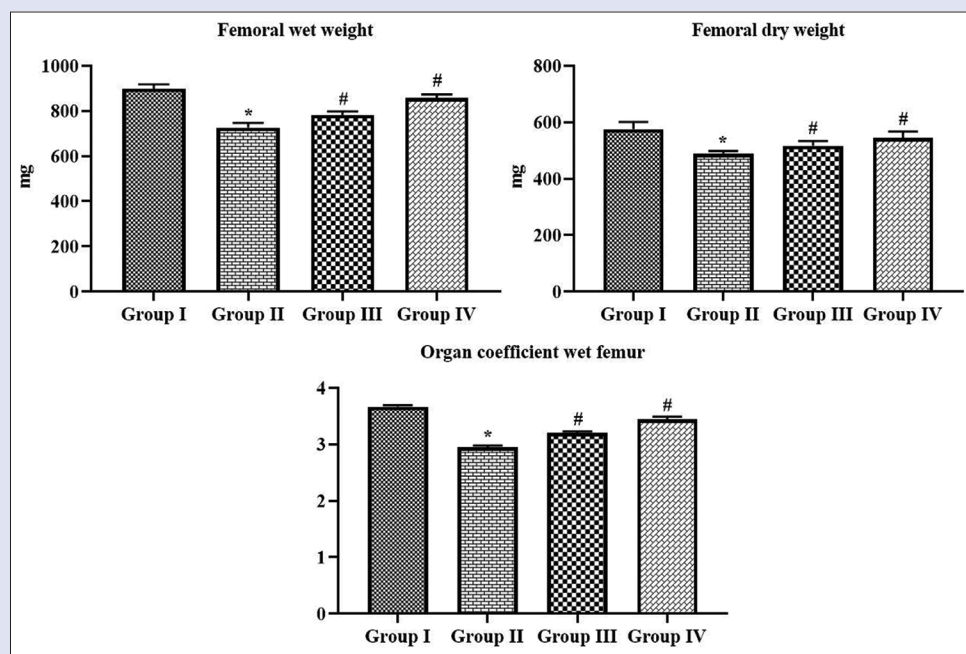


Figure 2: Efficacy of ononin in femoral wet and dry weight and organ coefficient of OVX rats. Group I—sham (SHAM) operated control rats, group II—ovariectomized (OVX) rats, group III—OVX rats treated with 10 mg/kg body weight of ononin, and group IV—OVX rats treated with 20 mg/kg body weight of ononin. Data was represented as the mean $\pm$ SD of triplicates ($n = 6$). * $P < 0.05$ —control compared with other groups and ** $P < 0.05$ —OVX-induced group compared with other groups

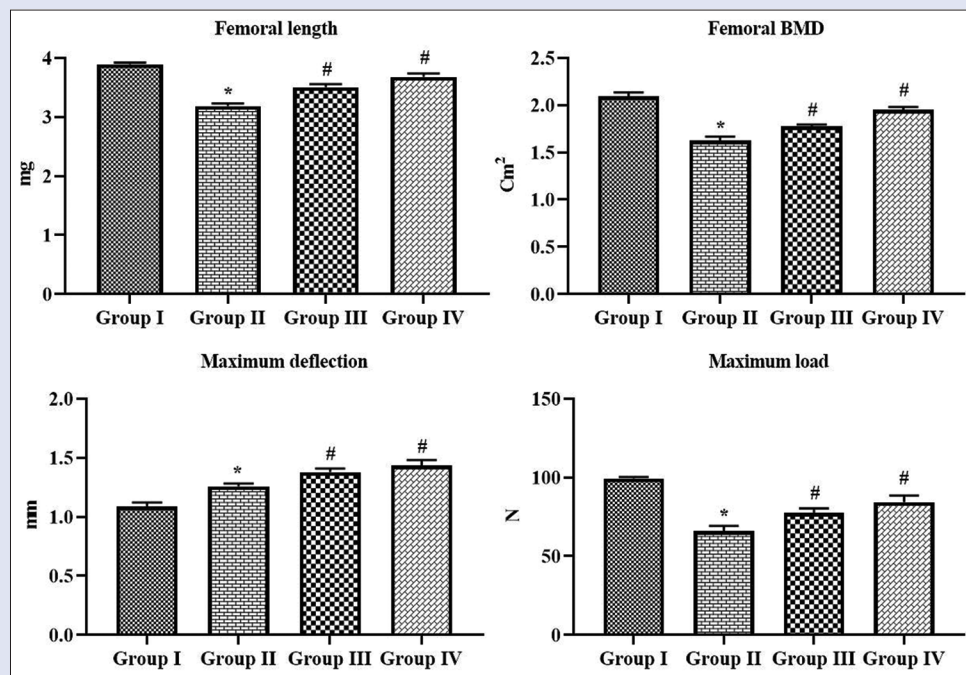


Figure 3: Efficacy of ononin in femoral fitness and biomechanical properties of OVX rats. Group I—sham (SHAM) operated control rats, group II—ovariectomized (OVX) rats, group III—OVX rats treated with 10 mg/kg body weight of ononin, and group IV—OVX rats treated with 20 mg/kg body weight of ononin. Data was represented as the mean $\pm$ SD of triplicates ($n = 6$). * $P < 0.05$ —control compared with other groups and ** $P < 0.05$ —OVX-induced group compared with other groups

evaluated using biomechanical parameters such as maximum load and deflection. Both maximum load and deflection were low in OVX animals when compared with control animals. Ononin (10 and 20 mg/kg body weight) significantly improved the biomechanical properties of OVX rats.

Efficacy of ononin on femoral diaphysis of OVX rats

Next, we evaluated the efficacy of ononin on femoral diaphysis by assessing the energy, stiffness, modulus, and stress in experimental animals [Figure 4]. These factors were unchanged in control animals.

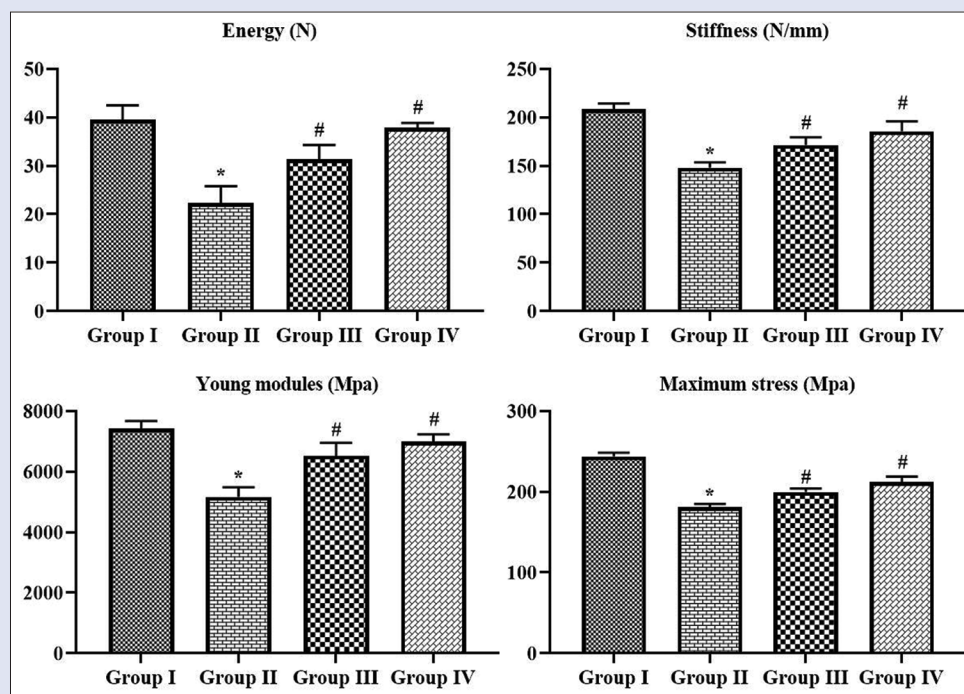


Figure 4: Efficacy of ononin on femoral diaphysis of OVX rats. Group I—sham (SHAM) operated control rats, group II—ovariectomized (OVX) rats, group III—OVX rats treated with 10 mg/kg body weight of ononin, and group IV—OVX rats treated with 20 mg/kg body weight of ononin. Data was represented as the mean $\pm$ SD of triplicates ($n = 6$). * $P < 0.05$ —control compared with other groups and ** $P < 0.05$ —OVX-induced group compared with other groups

All these factors were lowered in OVX animals. In groups III and IV animals, ononin (10 and 20 mg/kg body weight) significantly increased stress, modulus, stiffness, and energy, which demonstrates the efficacy of ononin in OVX rats.

Potent role of ononin on lipid profile of OVX rats

Figure 5 shows the lipid profile such as TC, TG, HDL-C, and LDL-C of experimental animals. Noticeable dissimilarity was observed in serum levels of TC, HDL-C, LDL-C, and TG in ononin-treated and control animals. Ononin (10 and 20 mg/kg body weight) markedly decreased the levels of TC, TG, LDL-C, and HDL-C in OVX rats. Ononin also decreased the level of TG in group II animals. In addition to this, ononin also decreased the level of HDL-C in groups III and IV animals more than that of group I animals.

Mitigating the role of ononin in blood markers associated with the bone of OVX rats

Bone metabolism of OVX rats treated with ononin was assessed by evaluating the levels of blood markers [Figure 6]. The serum concentration of calcium, phosphorus, and creatinine was found to be reduced in OVX rats than that of control animals. Furthermore, the level of TRAP was increased in OVX rats compared to control animals. Ononin (10 and 20 mg/kg body weight) increased the levels of serum calcium, phosphorus, and creatinine in OVX rats (groups III and IV). It also reduced the TRAP level in groups III and IV animals.

Bone restoration and configuration were assessed in experimental animals by analyzing the levels of E2, BGP, and ACP [Figure 7]. Serum levels of E2, BGP, and ACP levels were upregulated in OVX rats than that of control animals. Ononin (10 and 20 mg/kg body weight) decreased the serum levels of E2, ACP, and BGP in groups III and IV animals.

Histopathological examination and biomechanical analysis of bone tissues of OVX rats treated with ononin

The efficacy of ononin on the trabecular structure of OVX rats was assessed using histopathological examination of the ipsilateral femur by H and E staining [Figure 8]. Trabeculae were lowered in OVX rats than in control animals together with destruction of reticulate arrangement. Numerous trabecular interconnections were found after the administration of ononin. Broader trabeculae with the lesser spatial arrangement were seen in OVX animals treated with 20 mg of ononin than that of 10 mg ononin in group III rats. Therefore, the histopathological examination of vaginal tissues of control and OVX rats exhibited the potential defensive action of ononin in rats induced with OVX. Ononin prevented vaginal and uterine atrophy induced by OVX. In addition to this, the maximum fracture load given to the femoral midshaft [Figure 9] and fracture load imposed on the femoral neck [Figure 9] was augmented following administration of ononin in OVX rats.

Augmented efficacy of ononin on morphological parameters of OVX rats

Osteoporotic features such as trabecular number, area, and thickness were markedly decreased and trabecular separation was noticeably increased in ovariectomized rats [Figure 10]. OVX rats administered with ononin (10 and 20 mg/kg body weight) displayed improved thickness, area, and a number of trabeculae together with the reduction in trabecular separation.

Efficacy of ononin on levels of OPG/RANKL in OVX rats

The mRNA expression of OPG was significantly lowered and mRNA expression of RANKL was upregulated in OVX rats than that of control

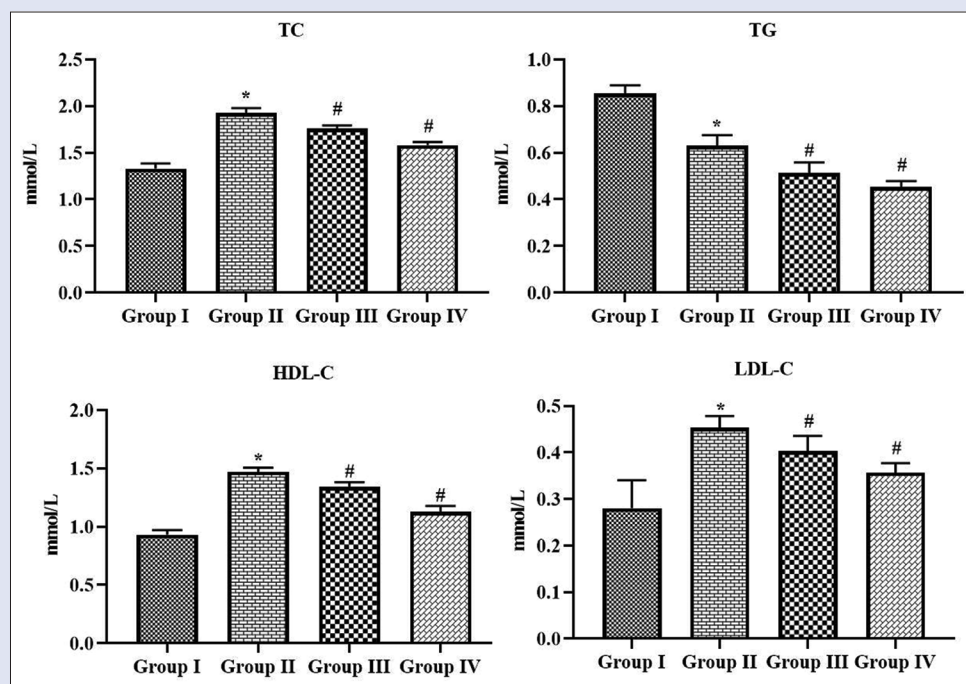


Figure 5: Potent role of ononin on lipid profile of OVX rats. Group I—sham (SHAM) operated control rats, group II—ovariectomized (OVX) rats, group III—OVX rats treated with 10 mg/kg body weight of ononin, and group IV—OVX rats treated with 20 mg/kg body weight of ononin. Data was represented as the mean $\pm$ SD of triplicates ($n = 6$). * $P < 0.05$ —control compared with other groups and ** $P < 0.05$ —OVX-induced group compared with other groups. TC = Total cholesterol; TG = Triglyceride; HDL-C = High-density lipoprotein cholesterol; LDL-C = Low-density lipoprotein cholesterol

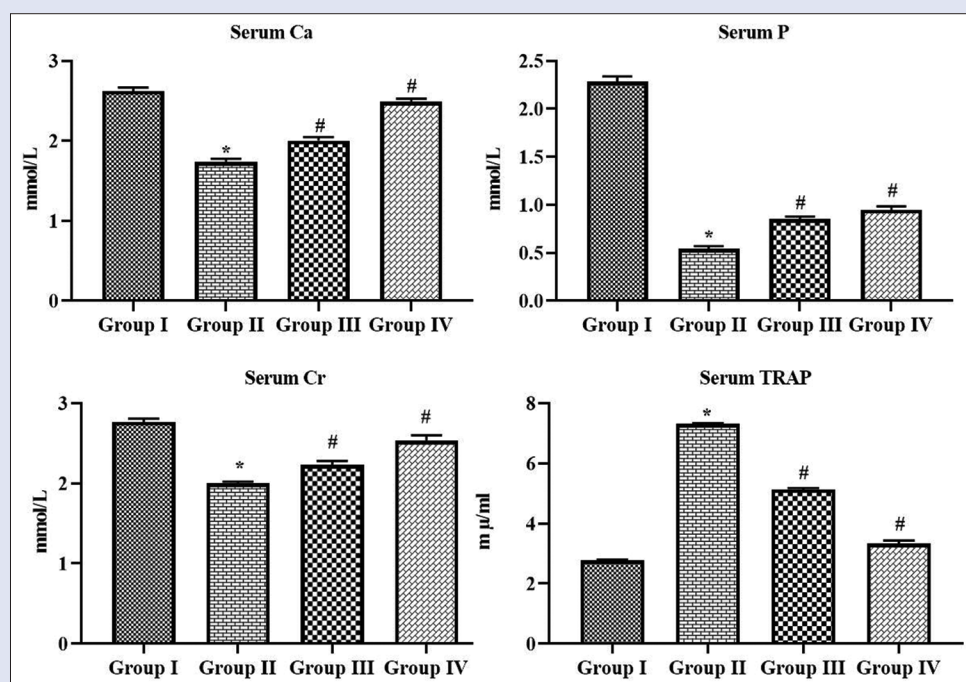


Figure 6: Mitigating role of ononin in blood markers associated with bone of OVX rats. Group I—sham (SHAM) operated control rats, group II—ovariectomized (OVX) rats, group III—OVX rats treated with 10 mg/kg body weight of ononin, and group IV—OVX rats treated with 20 mg/kg body weight of ononin. Data was represented as the mean $\pm$ SD of triplicates ($n = 6$). * $P < 0.05$ —control compared with other groups and ** $P < 0.05$ —OVX-induced group compared with other groups. Ca = Calcium; P = Phosphorus; Cr = Creatinine; TRAP = Tartrate-resistant acid phosphatase

group animals. The mRNA expression of OPG/RANKL was augmented significantly following ononin administration. Ononin downregulated

the mRNA level of RANKL and upregulated the level of OPG in groups III and IV animals [Figure 11].

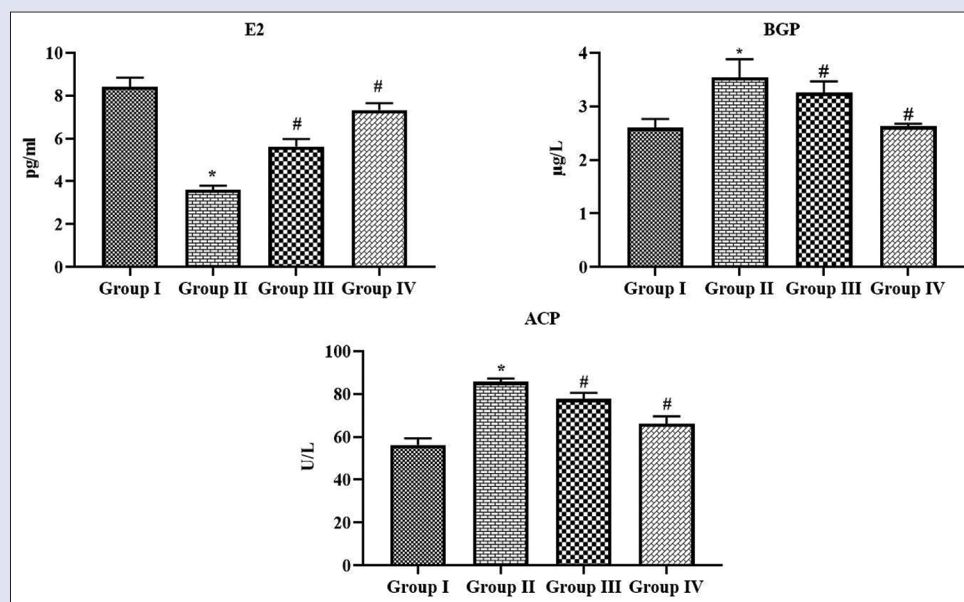


Figure 7: Mitigating role of ononin in blood markers associated with bone of OVX rats. Group I—sham (SHAM) operated control rats, group II—ovariectomized (OVX) rats, group III—OVX rats treated with 10 mg/kg body weight of ononin, and group IV—OVX rats treated with 20 mg/kg body weight of ononin. Data was represented as the mean $\pm$ SD of triplicates ($n = 6$). * $P < 0.05$ —control compared with other groups and ** $P < 0.05$ —OVX-induced group compared with other groups. E2 = Estradiol; BGP = Bone gla protein; ACP = Acid phosphatase

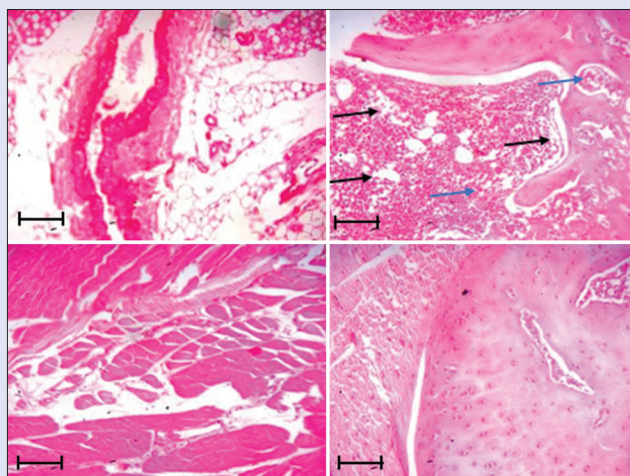


Figure 8: Histopathological examination of ipsilateral femurs of OVX rats treated with ononin (H&E staining). Group I—sham (SHAM) operated control rats showed normal structures, group II—ovariectomized (OVX) rats showed reduced trabeculae (blue arrows) and bone tissue destructions (black arrows), group III—OVX rats treated with 10 mg/kg body weight of ononin, and group IV—OVX rats treated with 20 mg/kg body weight of ononin showed ameliorative effects. Data was represented as the mean $\pm$ SD of triplicates ($n = 6$). * $P < 0.05$ —control compared with other groups and ** $P < 0.05$ —OVX-induced group compared with other groups. Scale bar = 50 μ m, Magnification = 40 $\times$

Role of ononin in the expression of Runx2, Osx, and ALP in OVX rats

Expression of ALP, Runx2, and Osx was lowered in OVX rats than that of control animals. Ononin (10 and 20 mg/kg body weight) upregulated the expression of Osx, Runx2, and ALP in groups III and IV animals. The mRNA expression of Runx2, Osx, and ALP

found in group IV animals was comparable to that of control group animals [Figure 12].

Effect of ononin in the suppression of serum proinflammatory markers of OVX rats

Proinflammatory cytokines such as IL-1 β , TNF- α , and IL-6 were responsible bone desorption. OVX rats showed marked upregulation in levels of IL-1 β , TNF- α , and IL-6 in the serum of group II animals [Figure 13]. Ononin (10 and 20 mg/kg body weight) treatment significantly lowered the levels of IL-1 β , TNF- α , and IL-6 in groups III and IV animals, thereby stimulating the bone metabolism progression.

DISCUSSION

Osteoporosis is a progressive bone disorder characterized by a reduction in bone density and mass and changes in the bone architecture which increases the risk of fracture. It affects approximately 200 million of people worldwide and disease risk is increased as age progresses. In addition to fractures, individuals also develop numerous secondary health problems resulting in death.^[54,55] Flavonoids such as formononetin and ononin have demonstrated antineoplastic,^[30,31] antimicrobial,^[32] antioxidant,^[33,34] antiarthritic,^[35] antiinflammatory,^[36,37] vasorelaxant,^[38] cardioprotective,^[56] hypolipidemic,^[39] antitumor,^[40] and neuroprotective^[41] effect. Ononin is the glycosylated derivative of formononetin which is reported to have a potent antiinflammatory effect against inflammation induced with lipopolysaccharide.^[42] Therefore, in this study, we aimed to identify the efficacy of ononin against OVX-induced osteoporosis in rats. OVX rats are the most widely used animal model for understanding postmenopausal osteoporosis. OVX rats show the weakness of the bone caused by the lack of E2. A previous study has reported that low levels of E2 results in increased body mass of rats, which indicates that postmenopausal women have a higher risk of gaining weight and might experience osteoporosis.^[57]

The body weight of ovariectomized rats was increased after 4–5 weeks of surgical procedure, whereas the control group rats were found to be normal.

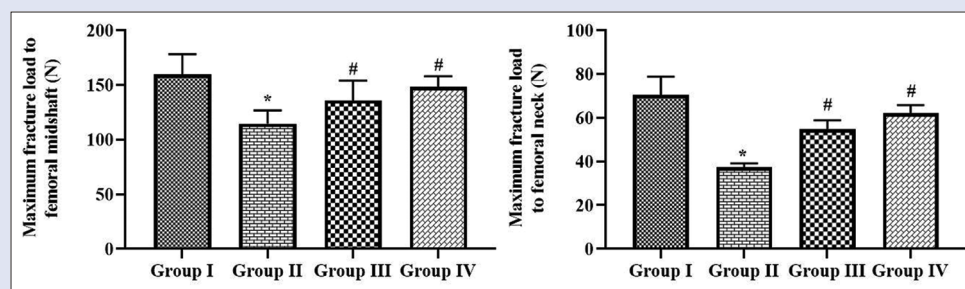


Figure 9: Biomechanical analysis of ipsilateral femurs of OVX rats treated with ononin. Group I—sham (SHAM) operated control rats, group II—ovariectomized (OVX) rats, group III—OVX rats treated with 10 mg/kg body weight of ononin, and group IV—OVX rats treated with 20 mg/kg body weight of ononin. Data was represented as the mean $\pm$ SD of triplicates ($n = 6$). * $P < 0.05$ —control compared with other groups and ** $P < 0.05$ —OVX-induced group compared with other groups

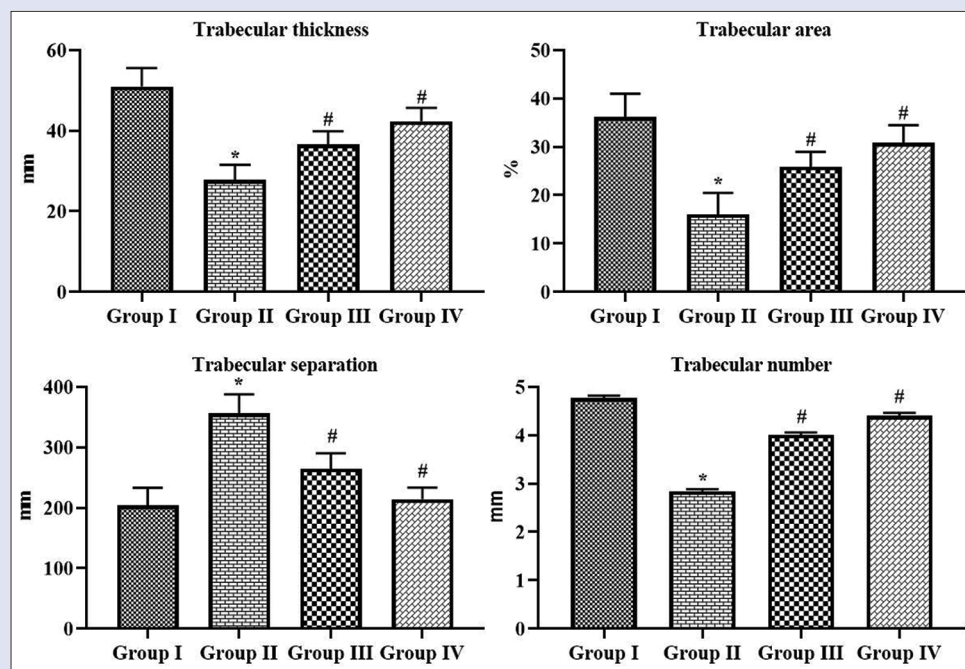


Figure 10: Augmented efficacy of ononin on morphological parameters of OVX rats. Group I—sham (SHAM) operated control rats, group II—ovariectomized (OVX) rats, group III—OVX rats treated with 10 mg/kg body weight of ononin, and group IV—OVX rats treated with 20 mg/kg body weight of ononin. For determining trabecular thickness, area, separation, and number image analysis software is used. Data was represented as the mean $\pm$ SD of triplicates ($n = 6$). * $P < 0.05$ —control compared with other groups and ** $P < 0.05$ —OVX-induced group compared with other groups

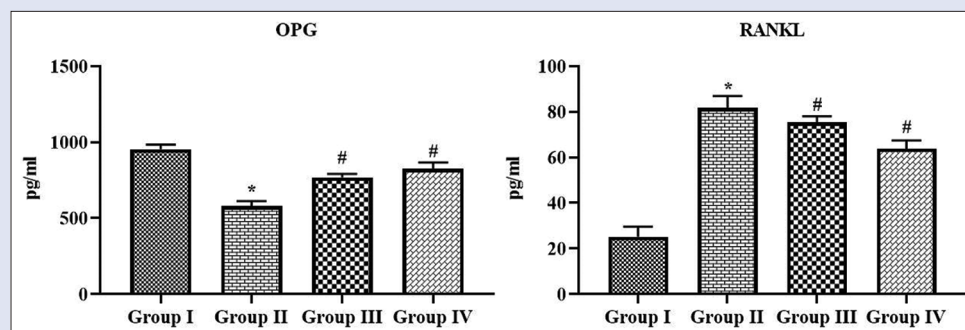


Figure 11: Efficacy of ononin on levels of OPG/RANKL in OVX rats. Group I—sham (SHAM) operated control rats, group II—ovariectomized (OVX) rats, group III—OVX rats treated with 10 mg/kg body weight of ononin, and group IV—OVX rats treated with 20 mg/kg body weight of ononin. Data was represented as the mean $\pm$ SD of triplicates ($n = 6$). * $P < 0.05$ —control compared with other groups and ** $P < 0.05$ —OVX-induced group compared with other groups

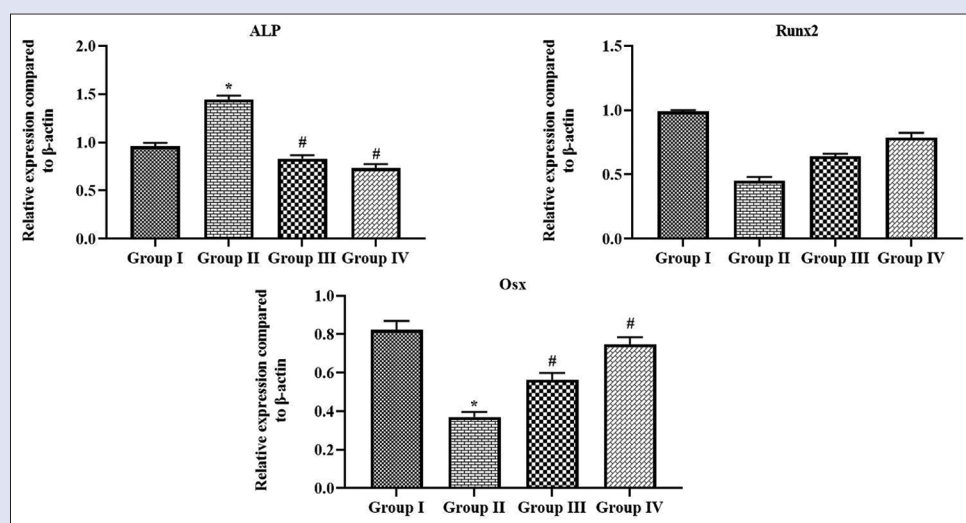


Figure 12: Potent role of ononin in Runx2, Osx, and ALP expression of OVX rats. Group I—sham (SHAM) operated control rats, group II—ovariectomized (OVX) rats, group III—OVX rats treated with 10 mg/kg body weight of ononin, and group IV—OVX rats treated with 20 mg/kg body weight of ononin. mRNA expression of Osx, Runx2, and ALP was analyzed using qRT-PCR and normalized to β -actin expression of control group. Data was represented as the mean $\pm$ SD of triplicates ($n = 6$). * $P < 0.05$ —control compared with other groups and ** $P < 0.05$ —OVX-induced group compared with other groups

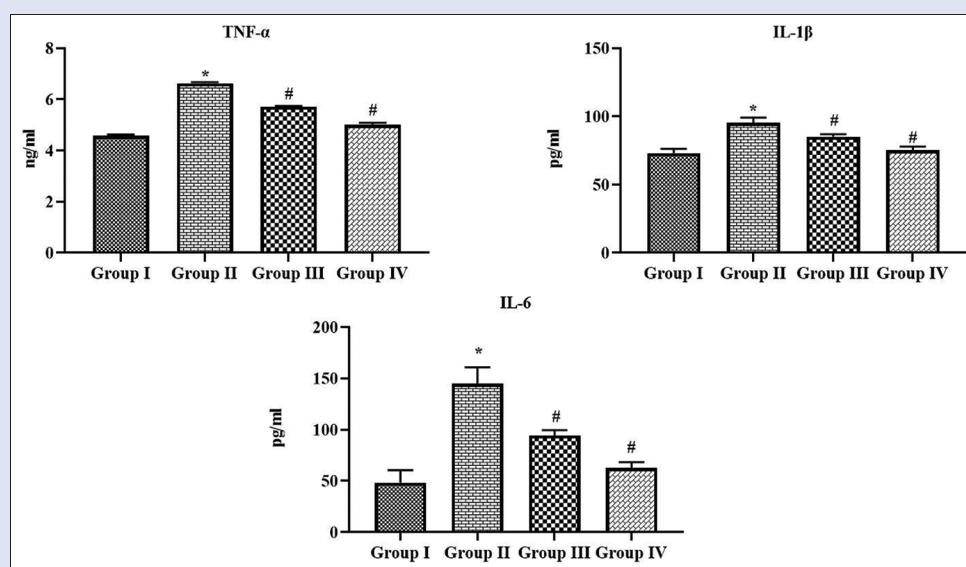


Figure 13: Effect of ononin in suppression of serum proinflammatory markers of OVX rats. Group I—sham (SHAM) operated control rats, group II—ovariectomized (OVX) rats, group III—OVX rats treated with 10 mg/kg body weight of ononin, and group IV—OVX rats treated with 20 mg/kg body weight of ononin. Data was represented as the mean $\pm$ SD of triplicates ($n = 6$). * $P < 0.05$ —control compared with other groups and ** $P < 0.05$ —OVX-induced group compared with other groups. TNF- α = Tumor necrosis factor- α ; IL-1 β = Interleukin-1 β ; IL-6 = Interleukin-6

Ononin decreased the weight gain induced by OVX in groups III and IV animals. Following the onset of osteoporosis, the absorption of lipid was lowered.^[58] Interestingly ononin treatment enhanced the lipid profiles such as TG, HDL-C, LDL-C, and TC in OVX rats.^[39] Insufficiency of E2 is most commonly observed during menopause, which causes the breakdown of calcium and hastens the process of bone degradation.^[59] In this study, loss of calcium in OVX rats was improved following ononin administration which correlates with a previous study, which reported increased excretion of calcium in women during E2 insufficiency.^[60] Remodeling of bones plays a vital role in the progression of bone metabolism, arrangement, and its necessity throughout an individual's lifetime. Osteoblasts and osteoclasts play an important role in bone metabolism. When the homeostatic balance

disrupts between arrangement and reestablishment, it ultimately results in bone loss which is an initiator of osteoporosis.^[61]

Modifications of bone were assessed using biomarkers such as ACP and ALP.^[11,57] In the case of E2 deficiency in rats, alterations in levels of ALP and ACP were observed, which agrees with the results of this study.^[62] OVX rats treated with ononin markedly brought about a reduction in ALP and ACP levels. However, the reduction brought about by a higher dose of ononin (20 mg) was markedly significant when compared with a lower dose of ononin (10 mg). These changes increase the risk of osteoporosis development.^[63,64] The serum levels of IL-6, TNF- α , and IL-1 β , which were noticeably higher in OVX rats but their levels were markedly reduced upon treatment with ononin. Thus, treatment with

ononin helped the ovariectomized rats to bring about a reduction in the levels of markers of inflammation.

Ononin in OVX rats showed less effect on organ mass and various other constants, which indicates minimal protective action in OVX-induced degeneration of the uterus and vagina. BMD, biomechanical aspects, arrangement of bones, and femoral strength are the indicators of bone strength.^[49] In this study, ononin enhanced the femoral measurement and BMD of OVX rats. It also augmented refraction of femur and weight of organ coefficients in OVX rats which correlates with earlier literature evidence.^[65] Furthermore, ononin protected the trabecular arrangement and cortical bones of ovariectomized rats. It did not exhibit a toxic outcome on the configuration of bones in ovariectomized rats. Cytokines such as RANKL and OPG are expressed by osteoblast cells which can stabilize the function, differentiation, and survival of osteoclasts.^[66] Active osteoclasts are formed when RANKL stimulates and localizes into receptors of adult osteoclasts. Osteoblasts trigger the receptor of RANKL and thus enhance RANKL attachment.^[67-69] In this study, RANKL expression was overexpressed in OVX rats, whereas OPG expression was reduced. Both high and low doses of ononin brought about the restoration of cytokine levels. Thus, the findings of this study show the efficacy of ononin in preventing osteoporosis among the other flavonoid compounds which can be used as a potent therapeutic agent for treating bone disorders.

CONCLUSION

To conclude, ononin was found to alleviate osteoporosis in OVX rats by bringing about an increment in body weight, restoring the levels of lipids, and retaining the uterus index. Ononin was also found to enhance ACP, E2, and BGP expression in OVX rats. It was also found to restore the serum levels of calcium, phosphorus, creatinine, TRAP, and markers of inflammation in OVX rats. Ononin has a potent role in the biomechanical properties and femoral stretch of rats that were ovariectomized. Hence, taken as a whole, the outcome of this study suggests that it can be used as a potent drug to treat disorders associated with bone. Furthermore, more studies are warranted in the future to understand the mechanistic role of ononin against the prevention of osteoporosis.

Author contributions

Lijun Tian, BaoShan Xu, and Tonghao Wang—conceptualization, experimental design, supervising, and manuscript draft. Lilong Du and Yongjin Li—preparation of manuscript and data interpretation and Guowang Li and Lijun Tian—project management.

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Conflicts of interest

There are no conflicts of interest.

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