Anti-osteoporosis Effect of Fisetin against Ovariectomy Induced Osteoporosis in Rats: *In silico*, *in vitro* and *in vivo* Activity

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Abstract: Osteoporosis is a bone related disease that is characterised by bone loss that further increases the susceptibility to bone fractures and bone frailty due to disturbances in the micro-architecture of bone tissue. Fisetin (flavonoids) exhibited anti-inflammatory and antioxidative stress effects against various diseases. In this protocol, we make an effort to comfort the anti-osteoporosis effect of fisetin against ovariectomy (OVX) induced osteoporosis. A docking study of fisetin and alendronate on the estrogen (α and β) and vitamin D receptors was carried out. SaOS-2 (osteoblast like human) cells were used for the estimation of cell proliferation. The OVX induced OVX model was used and three doses of fisetin and alendronate was given to rats till 16 weeks. The hormone levels, bone turnover markers and biochemical parameters were estimated. Fisetin was docked into estrogen (α and β) and vitamin D receptors, resulting in stable complexes with lower binding scores. Fisetin significantly (*p* < 0.001) exhibited the induction of cell proliferation against the SaOS-2 cells. OVX induced osteoporosis rats exhibited a suppression of body weight and uterus index, after the Fisetin treatment. Fisetin treatment significantly (*p* < 0.001) improved the level of bone mineral content (BMC), bone mineral density (BMD) and biochemical parameters such as energy, maximum load, stiffness, young modules, maximum stress and reduced the level of 1,25(OH)₂D₃ and E₂. Fisetin treatment significantly (*p* < 0.001) declined the level of phosphorus (P), calcium (Ca) and boosted the level of VitD. Fisetin treatment significantly (*p* < 0.001) reduced the malonaldehyde (MDA) level and enhanced the glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) level in the bone, intestine and hepatic tissue. Fisetin treatment suppressed the cytokines, RANKL/OPG ratio, receptor activator of nuclear factor-κB ligand (RANKL) and improved the level of osteoprotegerin (OPG). The findings suggest that fisetin could be a beneficial phytoconstituent for the treatment and prevention of postmenopausal osteoporotic complications.

Key words: fisetin, postmenopausal osteoporosis, hormones, receptor activator of nuclear factor-κB ligand, calcium, Vitamin D

Abbreviations: OVX; Ovariectomy, BMC; Bone mineral content, BMD; Bone mineral density, P; Phosphorus, Ca; Calcium, GSH; Glutathione, CAT; Catalase, SOD; Superoxide dismutase, RANKL; Receptor activator of nuclear factor-κB ligand, OPG; Osteoprotegerin, ERT; Estrogen replacement therapy, HRT; Hormone replacement therapy, NF-κB; Nuclear factor-κB, MSCs; Marrow stomal cells, RAGE; Receptor for advanced glycation end products, TCA; Trichloroacetic acid, TBA; Thiobarbituric acid, OC; Osteocalcin, Uca; Urine calcium, UP; Urine phosphorus, ALN; Alendronate, BALP; Bone specific alkaline phosphatase, CTX; Cross-linked C-terminal telopeptides of type I, TRAcP 5b; Tartrate resistant acid phosphatase

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1 Introduction

Osteoporosis is a chronic and multifactorial bone disease that expends unnoticeably until the advanced stage with complex pathophysiology\(^1\). Osteoporosis is the major common health problem in the geriatric population worldwide\(^2\),\(^3\),\(^4\). Every year, 1.24 million fractures caused by osteoporosis are reported worldwide. In the next 30 years, the incidence of hip fractures in both men and women will increase by 240% and 310%, respectively. The incidence of osteoporosis is increasing in Asia day by day, especially in China. A clinical study showed that postmenopausal women have osteoporosis and osteopenia\(^5\),\(^6\),\(^7\). The incidence of osteoporosis increases in men at the age of 20 to 89 years old. Osteoporosis is mainly categorised by the suppression of bone mass and density, which results in enhanced fragility and fracture of bones\(^2\),\(^3\),\(^7\). It is a major health problem in women, especially the elderly and postmenopausal women. Menopause in those women is related to an enhanced risk of osteoporosis, which creates an imbalance between the osteoclast resorption and formation of bone due to loss of hormones such as estrogen (E\(_2\))\(^2\),\(^3\),\(^8\),\(^9\). The reduction of E\(_2\) plays a crucial role in the inhibition of bone density loss and ensuing the osteoporosis.

The current available therapies for osteoporosis in women (elderly and postmenopausal) are estrogen replacement therapy (ERT) and hormone replacement therapy (HRT)\(^2\),\(^3\),\(^8\),\(^9\). However, there are limitations to the therapy due to adverse/side effects, such as long-term HRT therapy increasing the risk of endometrial, ovarian, and breast cancer\(^5\),\(^11\),\(^12\). The levels of vitamin D and calcium are altered during osteoporosis, and studies suggest that avoiding smoking, alcohol, caffeine, and regular exercise can help prevent the development of osteoporosis. Another thing, regular intake of a controlled diet (rich in vitamin D and Ca) helps in reducing bone density and loss. Bone loss occurred in postmenopausal women due to increased oxidative stress and inflammatory reactions, which started due to the continuous production of free radicals and modulated the level of endogenous antioxidants and boosted the inflammatory parameters\(^13\). Oxidative stress promotes the osteoclastic function and bone differentiation of bones and starts bone loss\(^8\),\(^9\).

The treatment of osteoporosis was very tedious a few years ago. Now, with the advancement of the science, there is an easy way to treat osteoporosis. Moreover, the recent treatments have limitations such as long-term safety and efficacy\(^1\),\(^14\). Currently, hormones and estrogen replacement therapy have various nonskeletal effects, including stroke, breast cancer and heart disease. Long term bisphosphonate treatment is used for the treatment of osteoporosis, but the treatment has adverse effects such as atypical fractures and osteonecrosis of the jaw. Parathyroid hormones (newer anabolic agents) are used for the treatment of osteoporosis\(^15\). Furthermore, the cost of the treatment and daily need for the injection have a limit for prolonged and widespread use. Moreover, some plants or herbs have a protective effect against bone loss due to its estrogen like effects but also contain a little amount of estrogen, it may also hint at the dangers of unidentified, unknown estrogen\(^2\). Therefore, the researchers continue searching for a cheaper and effective drug with less or no side effect to improve upon the present therapies still show a definite need.

Excessive osteoclast resorption and formation are considered as the significant pathological alteration in OVX induced osteoporosis. Osteoclast precursor cells express the receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL), which binds to RANKL receptors on the surface of osteoclast precursors. RANKL-osteoprotector (OPG) isolated from osteoblasts and narrow stomal cells (MSCs), opposed the effect. Estrogen deficiency enhance the OPG production and suppress the RANKL source, thereby arbitrating osteoclast function and formation\(^16\). Estrogen also boost the production of osteoclastogenic cytokines, which act as pro-resorptive factors via boosting the expression of RANKL in osteoblast lineage cells\(^17\). Flavonoids are the low molecular weight polyphenolic phytoconstituent commonly synthesized in lot of plants\(^18\),\(^19\). Fisetin (IUPAC name-3',4',7-trihydroxyflavone), flavonoid, commonly found in various vegetable and fruits such as grape, cucumber, strawberry, onion and persimmon\(^20\),\(^21\). Fisetin involved in balancing the various oxidative stress aspects viz., anti-liperoxidation effect or scavenging the free radicals in the biological system\(^20\),\(^22\). The capability of fisetin to scavenge the free radicals exhibited its antioxidant effect and biological effects. Fisetin exhibited the wide range of pharmacological effects such as antioxidant, neuroprotective, anti-tumor, anti-inflammatory and neurotrophic\(^22\),\(^23\). Fisetin also suggest the neuroprotective and chemotherapeutic effect in human and mice. As hydrophobic agent, fisetin easily infiltrate into cell membranes and accumulate in cells to exert its neuroprotective, neurotrophic and antioxidant effects\(^24\),\(^25\).

Previous report suggests that fisetin treatment could relieve inflammation, cell apoptosis and oxidative stress via suppression the receptor for advanced glycation end products (RAGE)/nuclear factor-κB (NF-κB) signaling\(^21\),\(^25\). Thus, this experimental protocol scrutinizes the anti-osteoporosis effect against SAOS-2 (osteoblast like human) cells and ovariectomy induced osteoporosis model in female rats and explore the underlying mechanism.

2 Materials and Methods

2.1 Chemicals

Fisetin (98%), alendronate sodium salt, trichloroacetic
acid (TCA) and 2-thiobarbituric acid (TBA) were purchased from the Sigma Aldrich (St. Louis, USA). All the reagents and chemicals used in this experimental study was analytical grade.

2.2 In silico study

We used molecular docking simulations at the Estrogen Alpha receptor catalytic ligand binding site to better understand the binding mode of (Fisetin) at the molecular level (PDBID: 5wgd). The docking of fisetin was done with Maestro, a Schrödinger software suite programme, version 9.6. Using the build panel, the ligand was sketched in 3D format and prepped for docking using the ligprep tool. The protein for the docking investigation was obtained from the Protein Data Bank (PDB ID: 5wgd) and synthesised by removing the solvent, adding hydrogen, and then using the Protein Preparation Wizard to minimise energy. The catalytic domain was used to create grids for molecule docking. Glide extra-precision (XP) mode was used to dock fisetin, with up to three postures preserved per molecule.

2.3 MM/GBSA study

MM/GBSA energy calculations implemented in the Prime module of the Schrödinger molecular modelling package were used to investigate the free binding energy of complexes of Fisetin and Alendronate with Estrogen Alpha.

2.4 ADMET analysis

Schrödinger’s QikProp (Version 3.5) was used to perform ADMET analysis on fisetin and alendronate. It provides comparison ranges for comparing a molecule’s attributes to those of 95% of known medications. The partition coefficient, human oral absorption, CNS (central nervous system) activity, and gut–blood barrier permeability were the descriptors calculated.

2.5 In vitro model

2.5.1 Cell proliferation

The effect of Fisetin and ALN on the cell proliferation was estimated on the human SAOS-2 osteosarcoma cell lines (osteoblast like effect). Briefly, the cells were cultured in the McCoy’s medium contained foetal bovine serum (15%). The cells cultured in the medium was incubated under standard condition such as temperature (37°C) and humidified CO₂ atmosphere (5%) [19].

2.5.2 MTT assay

MTT assay was used for the determination of cell viability effect of Fisetin on metabolic activity of SAOS-2. The cells (100 μl/well) were harvested and seeded in 96-well plates with a density of 1.5 × 10⁴/mL and cells were kept for incubation for 24 h. After the incubation, the different concentration of Fisetin and ALN was treated with the cells [19].

| S. No | Group       | Treatment |
|-------|-------------|-----------|
| 1     | Normal control | Saline    |
| 2     | OVX         | Saline    |
| 3     | OVX + Fisetin | 10 mg/kg |
| 4     | OVX + Fisetin | 20 mg/kg |
| 5     | OVX + Fisetin | 30 mg/kg |
| 6     | OVX + Fisetin | 2.5 mg/kg |

2.6 Experimental animals

Sprague-Dawley (aged-3 month old, sex-female; weight 200-230 g) were procured from the departmental animal house and housed under the controlled laboratory condition such as 20 ± 5°C temperature, 65% relative humidity and 12/12 h light/dark cycle. The rats were fed with standard pellet diet and water ad libitum. Before start the experimental protocol, the rats were kept in the 7 days for acclimatization for adopting the laboratory condition.

2.7 Experimental design

The rats were divided into 6 groups and each groups contains 6 rats. Table 1 showed the experimental groups. The rats were received the oral administration of above treatment for 8 weeks, respectively.

The rats were starved overnight at the end of the experiment, and urine from all groups was collected via micturition induced by manual pressure and stored at −20°C for further analysis. The rats were anaesthesia using the ketamine and blood was collected from the dorsal aorta in the pre-incubated tubes and centrifuged at 1000 g for 10 min to separate the serum. The absolute weight of uterine and thymus was estimated and normalized with body weight using the following formula:

Relative weight of uterus = Weight of uterus per 100 g of body weight

The femoral neck was further processed for performing the mechanical testing. The left femur and vertebra bone were used for estimation the mineral content [26].

2.8 Bone marker parameters

The osteocalcin EIA kit was used to calculate the amount of osteocalcin (OC) based on the manufacturer's instructions (Xinqidi Bio Technology, Inc, China). The urine deoxypyridinoline (DPD) level was determined using a competitive enzyme immunoassay in a microassay stripwell model (Quidel, Mountain View, CA, USA) as directed by the manufacturer. Acid phosphatase (ACP) and Beta-CrossLaps (β-CTX) were estimated using the immunoassay analyser (Cobas-Roche, Basel, Switzerland) following the
manufactured instruction.

2.9 Urine parameters
Urine calcium (UCa) and Urine phosphorus (UP) level was estimated using the commercial colorimetrical kits (Boehringer Mannheim GmbH) following the manufacture description.

2.10 Hormone estimation
The serum hormones E2 was estimated using the radioimmunoassay (Subio, Inc., China).

2.11 Serum phosphorus (P) and calcium (Ca) level
Ammonium molybdate and arsenazo-3 dye colorimetric model was used for the estimation of P and Ca level using the previous method with minor modification26.

2.12 Plasma enzymes
The level of Bone specific alkaline phosphatase (BALP), Cross-linked C-terminal telopeptides of type I (CTX) and Tartrate resistant acid phosphatase (TRACP 5b) were estimated using the nitrophenol based method using the previous reported method with minor modification24.

2.13 BMD and BMC
Lunar prodigy advance dual energy X ray absorptiometry was used for the estimation the level of BMD and BMC with minor modification of previous reported method26.

2.14 Statistical analysis
The data were showed as mean ± S.E.M. Comparisons between the groups were made using the two-way analysis of variance using the Dunnett’s. Statistical analysis was carried out using the Graphpad Prism 7 (St. Louis, USA). The level of significance was showed as p < 0.05.

3 Results
3.1 Molecular docking of the vitamin D receptor (VDR) and oestrogen receptors (ERs)
Fisetin docking studies were carried out in order to gain a better understanding of Estrogen Alpha potency at the molecular level and shed light on interactions in the active site. The docking study of fisetin and alendronate on the estrogen alpha receptor. a: Binding interaction of compound fisetin against Estrogen alpha (Pdbid-5wgd), b: Ligplot interaction of drug fisetin against Estrogen Alpha (Pdbid-5wgd), c: Binding interaction of standard drug alendronate against Estrogen Alpha (Pdbid-5wgd), d: Ligplot interaction of standard drug Alendronate against Estrogen Alpha (Pdbid-5wgd), and e: superimposition of compound fisetin (brown) with Alendronate (green) against Estrogen Alpha (PDBID: 5WGD).

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Fig. 1 The docking study of fisetin and alendronate on the estrogen alpha receptor. a: Binding interaction of compound fisetin against Estrogen alpha (Pdbid-5wgd), b: Ligplot interaction of drug fisetin against Estrogen Alpha (Pdbid-5wgd), c: Binding interaction of standard drug alendronate against Estrogen Alpha (Pdbid-5wgd), d: Ligplot interaction of standard drug Alendronate against Estrogen Alpha (Pdbid-5wgd), and e: superimposition of compound fisetin (brown) with Alendronate (green) against Estrogen Alpha (PDBID: 5WGD).
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The re-docking of the reference ligand (Alendronate) into the active site of the VitD enzyme indicates that it binds to the same binding pocket, indicating that the current docking methodology is valid. The molecule fisetin binds securely in the active region of the VitD enzyme, according to docking results. This molecule fills the receptor cavity completely and forms a hydrogen bond with Ser237. In addition, when compared to normal alendronate, it forms hydrogen bonds with Ser237 and Tyr143 (Fig. 3).

3.2 MM/GBSA study

The stability of the ligands in the binding pocket of the Estrogen Alpha receptor was investigated using molecular mechanics/generalized born surface area (MM/GBSA). MM/GBSA tests were performed on compound fisetin. Fisetin has a binding free energy of 7.52 kcal, whereas the reference chemical (Alendronate) has a binding free energy of 2.21 kcal, demonstrating that Fisetin is stable in the oestrogen alpha receptor pocket (Table S1).

The stability of the ligands in the binding pocket of the VitD receptor was investigated using molecular mechanics/generalized born surface area (MM/GBSA). MM/GBSA tests

Fig. 2 The docking study of fisetin and alendronate on the estrogen beta receptor. a: Binding interaction of compound fisetin against Estrogen beta (Pdbid-3OLS), b: Ligplot interaction of drug fisetin against Estrogen beta (Pdbid-3OLS), c: Binding interaction of standard drug alendronate against Estrogen beta (Pdbid-3OLS), d: Ligplot interaction of standard drug Alendronate against Estrogen beta (Pdbid-3OLS) and e: superimposition of compound fisetin (brown) with Alendronate (green) against Estrogen beta (PDBID: 3OLS).
were performed on compound fisetin. Fisetin has a binding free energy of 2.15 kcal, whereas the reference chemical (Alendronate) has a binding free energy of 0.145 kcal, demonstrating that Fisetin is stable in the VitD receptor pocket (Table S2).

3.3 ADMET analysis
The ADMET studies of fisetin and alendronate were performed using Schrödinger’s Qikprop programme. Table S3 summarises the findings.

The ADMET studies of fisetin and alendronate were performed using Schrödinger’s Qikprop programme. Table S4 summarises the findings.

3.4 Cell viability
Figure 4 shows the effect of fisetin and ALN on the cell viability of SAOS-2 celllines. ALN (standard drugs) did not show any impact on the cell proliferation. On the other hand, fisetin treatment significantly showed the alteration in a dose dependent manner on metabolic activity. Fisetin higher doses exhibited the most pronounced inhibitory effects and showed the potential effect on osteoblastic activity.

3.5 Body weight and uterus index
Body weight of the control group increases in a normal pattern. OVX induced osteoporosis rats exhibited enhanced body weight as compared to their initial body weight and normal and treated rats. OVX treated rats received fisetin and ALN treatment significantly suppressed their body weight as compared to the OVX control group. The body weight of fisetin and ALN treated
Fig. 5  The effect of fisetin alendronate on the body weight and uterus index in OVX induced osteoporosis. **a**: body weight and **b**: uterus index. Treatment groups mention in Table 1. All the data showed as mean ± SEM. The comparison was performed between the OVX control and tested group rats using the Dunnett’s multiple comparison test. Where * $p < 0.05$ (significant), ** $p < 0.01$ (more significant) and *** $p < 0.001$ (extreme significant).

Fig. 6  The effect of fisetin alendronate on the level of BMC and BMD in OVX induced osteoporosis. Treatment groups mention in Table 1. All the data showed as mean ± SEM. The comparison was performed between the OVX control and tested group rats using the Dunnett’s multiple comparison test. Where * $p < 0.05$ (significant), ** $p < 0.01$ (more significant) and *** $p < 0.001$ (extreme significant). BMC were estimated (g) and BMD (g/cm$^2$). Where BMC; Bone mineral content, BMD; Bone mineral density, ALN; Alendronate.

Fig. 7  The effect of fisetin alendronate on the level of biochemical parameters in OVX induced osteoporosis. Treatment groups mention in Table 1. All the data showed as mean ± SEM. The comparison was performed between the OVX control and tested group rats using the Dunnett’s multiple comparison test. Where * $p < 0.05$ (significant), ** $p < 0.01$ (more significant) and *** $p < 0.001$ (extreme significant). Energy (N.mm), Maximum load (N), Stiffness (N/mm), Young modulus (MPa), Maximum stress (MPa). OVX; Ovariectomy, ALN; Alendronate.
rats was boosted as compared to their initial body weight (Fig. 5a).

The uterus index was increased in the osteoporosis control rats, but it was significantly reduced ($p < 0.001$) in the fisetin and ALN treated rats (Fig. 5b).

### 3.6 BMC and BMD

OVX induced osteoporosis rats showed a decreased level of BMC and BMD. Fisetin treatment significantly ($p < 0.001$) increased the level of BMC and BMD. Fisetin (30 mg/kg) showed the maximum up-regulation of BMC and BMD levels. ALN treatment showed the up-regulation of BMC and BMD levels (Fig. 6).

### 3.7 Biomechanical parameters

Biomechanical parameters such as energy, maximum load, stiffness, young modules and maximum stress (Fig. 7). OVX rats exhibited a reduction in the biomechanical parameters and fisetin treatment significantly ($p < 0.001$) increased the level of biomechanical parameters.

### 3.8 $E_2$, $1,25(OH)_2D_3$, FSH and LH

Fisetin and ALN treatment significantly ($p < 0.001$) reduced the level of $E_2$, $1,25(OH)_2D_3$, FSH, and LH in OVX-induced rats (Fig. 8).

### 3.9 Ca, P and Vit D

During osteoporosis, calcium and phosphorus levels decreased while vitamin D levels increased. OVX induced group rats showed similar results as compared to normal and other treated group rats. Fisetin significantly ($p < 0.001$) enhanced the level of Ca, P and reduced the level of Vit D (Fig. 9).

### 3.10 BALP, CTX and TRAcPs5b

OVX induced rats presented an augmented level of
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3.11 Urine parameters

Urine parameters such as Sca, SP, OC, ALP, Uca/Cr, DPD/Cr and UP/Cr presented in Fig. 11. OVX induced osteoporosis rats displayed an augmented level of urine parameter and fisetin treatment significantly ($p < 0.001$) reduced the level of urine parameters (Fig. 11).

3.12 Antioxidant parameters

Figure 12 showed the antioxidant parameters (MDA, SOD, GSH and CAT) in the bone, liver and intestine. OVX induced rats presented an augmented level of MDA (Fig. 12a) and reduced level of SOD (Fig. 12b), GSH (Fig. 12c) and CAT (Fig. 12d) in the bone, liver and intestine. Fisetin treatment significantly ($p < 0.001$) reduced the level of MDA and increased the levels of SOD, GSH and CAT in the bone liver and intestine.

3.13 Inflammatory cytokines

Figure 13 exhibited the effect of fisetin on the level of inflammatory cytokines. OVX induced osteoporosis rats exhibited an increased level of inflammatory cytokines and fisetin treatment significantly ($p < 0.001$) declined the level of inflammatory cytokines.
3.14 RANKL, OPG and RANKL/OPG ratio
OVX rats showed the increased level of RANKL, reduced level of OPG, and augmented the RANKL/OPG ratio (Fig. 14). Fisetin treatment significantly ($p<0.001$) reduced the level of RANKL, increased the level of OPG and reduced the ratio of RANKL/OPG.

4 Discussion
According to a previous study, ovariectomy reduces blood flow by lowering endothelial dysfunction and erythropoietic marrow levels. Reduced blood flow appears to play a key role in lowering BMD, according to both experimental and clinical evidence. Reduced estradiol levels in postmenopausal women impede bone growth and eventually contribute to osteoporosis. It is well documented that hypoxia significantly promotes the size and number of osteoclasts which results in enhanced...
inhibition and resorption of osteoblast activity\(^\text{13}\). Furthermore, plant drugs and plant isolated constituents that boost the blood supply to the bone tissue might be beneficial for the treatment of osteoporosis. In this experimental study, we used fisetin (a well-known phytoconstituent) to scrutinize the bone protective effect against the ovariectomy induced osteoporosis model in female rats.

Body weight increase during aging-related osteoporosis and women menopause is the main phenomenon\(^\text{2, 4}\). A similar result was observed in the osteoporosis control group rats. OVX rats exhibited enhanced body weight and reduced uterine index as compared to the control group rats. Previous research suggests that the decreased uterine weight in OVX-induced rats may be due to E\(_2\) deficiency\(^\text{2-5}\). Fisetin treatment significantly eased the excess gain in body weight and enhanced the uterine index in OVX induced osteoporosis rats. Fisetin treatment demonstrated a protective effect against fat accumulation, visceral fat and body weight gain, which correlates with our current findings.

OC, a biomarker of bone formation which is synthesised by osteoblasts and directly corresponds to their specific functions\(^\text{6, 14}\). Upregulated or boosted level of bone formation show the osteoporosis in postmenopausal or menopausal women and our findings coincide with this view \(\text{3, 28}\). In this study, the boost level of OC due to osteoblast formation in order to compensate for the bone loss induced due to E\(_2\) deficiency.

Osteoporosis is a condition caused by a balance or homeostasis between the formation and resorption of bones\(^\text{2, 3}\). In this experimental study, we scrutinised the inflammatory markers and bone turnover in the serum of experimental rats. In this study, we discovered that ovariectomy causes increased ROS deposition, which leads to an increase in inflammatory cytokines accumulation and oxidative stress, which increases bone loss and osteoclast generation. In postmenopausal women, the deterioration of bone function and composition, bone architecture is a common problem that further leads to the induction of osteoporosis. As people age, their bone structure, composition, and function often become impaired, leading to osteoporosis\(^\text{2-5}\). Bone is highly prone to the hormones dysfunction and age related loss, the current research focus is on scrutinising the protective effect of fisetin against OVX deficiency induced osteoporosis. During the postmenopausal period, the deficiency of E\(_2\) (ovary hormones) acts as a significant risk factor in the expansion of osteoporosis. Ovariectomy is a well-developed model for osteoporosis and it shows that the bone loss is similar to that observed in postmenopausal conditions\(^\text{4, 5, 28}\). This model is the most recommended for estimating the safety and effectiveness of therapies involved in the treatment of osteoporosis. OVX rats exhibited significant altered uterine, bone density, BMC, biochemical and other biochemical parameters due to E\(_2\) deficiency\(^\text{2-3}\).

Calcium is the essential nutrient for the growth of bone and teeth. Calcium plays a significant role in the maintenance and expansion of bone health and around 99% calcium is commonly present in the bones\(^\text{2}\). Some concentration of calcium is observed in the circulation, but the amount is low and stable. According to the previous research, the calcium concentration was around 0.85 mmol/L and 1.27 + 0.02 mmol/L in adult Wistar rats\(^\text{15}\). In this experimental study, we have found that the calcium concentration is almost near the previous studies. The calcium concentration was altered in the OVX group rats due to enhanced bone turnover following deprivation of estrogen. This is the important reason for induction the hypercalcemia. Another explanation for increasing calcium levels could be increased intestinal calcium absorption due to less antioxidant enzymes\(^\text{1, 2}\).

The absorption of intestinal calcium is influenced by

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**Fig. 14** The effect of fisetin alendronate on the level of RANKL, OPG and RANKL/OPG ratio in OVX induced osteoporosis. Treatment groups mention in Table 1. All the data showed as mean ± SEM. The comparison was performed between the OVX control and tested group rats using the Dunnett’s multiple comparison test. Where * \(p<0.05\) (significant), ** \(p<0.01\) (more significant) and *** \(p<0.001\) (extreme significant). Where RANKL (ng/mL) and OPG (ng/mL).

\(\text{RANKL; Receptor activator of nuclear factor-κB, OPG; Osteoprotegerin, OVX; Ovariectomy, ALN; Alendronate.}\)
GSH levels, according to previous research. GSH shortage can reduce intestinal calcium absorption by changing the mechanism and molecules involved in its transfer. Flavonoids have been shown in some studies to promote intestinal absorption and hence increase the bioavailability of micronutrients that are poorly or minimally absorbed, such as calcium. Some reported studies exhibit that flavonoids might enhance the body capacity to absorb the micronutrient (calcium). The result of present investigation showed that fisetin significantly boosts the concentration of GSH in the intestine and hepatic tissue, which in turn might enhance the calcium intestinal intake.

It is generally known that adequate vitamin D and calcium intake lessens the risk of osteoporosis. Cholecalciferol D₃ is essential for the absorption of P and Ca in the body, which is required for bone expansion. OVX induced osteoporosis rats exhibited altered levels due to deficiency of E₂ and Ca in the body, which is required for bone expansion. OVX absorption and bind to Vit D receptor exhibited that fisetin could affect estradiol synthesis or Ca absorption and increased the level of vitamin D.

In an experimental study, we observed that reduced levels of Vit D in the serum and fisetin treatment significantly increased the level of vitamin D.

The in silico target prediction by Schrödinger software exhibited that fisetin could affect estradiol synthesis or Ca absorption and bind to Vit D receptor. The binding capability of fisetin to the Vit D receptor was confirmed by the molecular docking study. The bone turnover marker includes CTX, ALP, and OC was boosted in the OVX group due to expansion of osteoporosis. Fisetin treatment significantly suppressed the level of bone turnover marker in the serum due to osteogenic and antioxidant effects. Fisetin suppressed the level of bone turnover marker due to its estradiol like effects. The molecular docking study of fisetin on VDR, ERα and ERβ exhibited that fisetin binds in a similar way as estradiol. Fisetin exhibited a lower score on the VDR, ERα and ERβ due to a lower number of hydrogen bonds with these receptors. Despite these in silico connections, the overall effects of fisetin were found to be inconsequential. Fisetin showed a protective effect against osteoporosis due to its antioxidant effect with a beneficial estrogenic effect, vitamin D and boosting the absorption of intestinal calcium. The RANKL/OPG axis is well established as a significant pathway in the regulation of homeostatic balance between bone resorption and formation. RANKL/OPG plays a significant role in arbitrating osteoclast differentiation. It is well known that OPG generated through osteoclast linkage and has a repressive effect on bone formation and RANKL is involved in bone fracture, which results in the formation of osteoclast. So, the ratio between the RANKL and OPG is necessary to estimate the bone integrity and density. We measured the levels of RANKL and OPG in the experimental rats, and found that OVX-induced rats had higher levels of RANKL and lower levels of OPG. Fisetin treatment significantly reduced the RANKL level and boosted the OPG level almost to control group rats. Consequently, the result suggests that the fisetin treatment considerably showed a protective effect against the OVX-induced bone loss via restoration of the level of RANKL/OPG.

It is well proven that postmenopausal women have higher levels of pro-inflammatory cytokines than women undergoing ERT. Previous research suggests that increased levels of vitamin D and E₂ initiate the production of cytokines. The boosted level of inflammatory cytokines, boost the production of free radicals and reduces bone formation and resorption. During osteoporosis, cytokines play a significant role in the regulation of bone turnover via increasing bone resorption. OVX-induced osteoporosis rats exhibited an enhanced level of cytokines (IL-1β, TNF-α and IL-6) and fisetin treatment significantly suppressed the production of cytokines.

5 Conclusion

The current research showed the anti-osteoporosis effect of fisetin against OVX-induced osteoporosis. An in silico study showed that fisetin docked to VDR (Vit D receptor) and estrogen receptors such as estrogen-α and estrogen-β. The docking study projected the VDR binding affinity and boosted the absorption of Ca. Fisetin treatment significantly maintains the level of phosphorus and calcium. Fisetin treatment significantly reduced the level of MDA and increased the level of SOD, CAT, GSH in the bone, liver and intestine. Fisetin improved the status of endogenous antioxidants and also helped with the absorption of intestinal Ca. Fisetin significantly reduced the level of inflammatory cytokines and inflammatory mediators, suggesting an anti-inflammatory effect. Fisetin also reduces osteoclast activity and suggesting an anti-inflammatory effect.

Author Contributions

Peng Feng performed the experimental protocol. Shijun Shu and Feifei Zhao analysed the biochemical data and performed the docking study. Feifei Zhao designed the experimental protocol and supervised the experimental study. All authors equally contributed in drafting and
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Supporting Information
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References
1) Guo, L.; Dang, M.; Song, Q.; Zhang, W.; Li, B. Protective effect of fucoxanthin on ovariectomy-induced osteoporosis in rats. *Pharmacogn. Mag.* **16**, 242-249 (2020).
2) Xu, H.; Liu, T.; Hu, L.; Li, J.; Gan, C. et al. Effect of caffeine on ovariectomy-induced osteoporosis in rats. *Biomed. Pharmacother.* **112**, 108650 (2019). doi: 10.1016/j.biopha.2019.108650
3) Zhang, Z.; Zhao, Q.; Liu, T.; Zhao, H. Effect of Vicenin-2 on ovariectomy-induced osteoporosis in rats. *Biomed. Pharmacother.* **129**, 110474 (2020). doi: 10.1016/j.biopha.2020.110474
4) Saleh, N.K.; Saleh, H.A. Olive oil effectively mitigates ovariectomy-induced osteoporosis in rats. *BMC Complement. Altern. Med.* **11**, 10 (2011). doi: 10.1186/1472-6882-11-10
5) Wang, Q.L.; Huo, X.C.; Wang, J.H.; Wang, D.P.; Zhu, Q.L. et al. Rutin prevents the ovariectomy-induced osteoporosis in rats. *Eur. Rev. Med. Pharmacol. Sci.* **21**, 1911-1917 (2017).
6) Wang, Y.F.; Xue, F. Effect of bushen jiangu decoction on ovariectomy-induced osteoporosis in rats. *Trop. J. Pharm. Res.* **18**, 327-331 (2019). doi: 10.4314/tjpr.v18i2.15
7) Xie, C.L.; Park, K.H.; Kang, S.S.; Cho, K.; Lee, D.H. Isoflavone-enriched soybean leaves attenuate ovariectomy-induced osteoporosis in rats by anti-inflammatory activity. *J. Sci. Food Agric.* **101**, 1499-1506 (2021). doi: 10.1002/jsfa.10763
8) Jung, M.Y.; Kim, J.W.; Kim, K.Y.; Choi, S.H.; Ku, S.K. Polycan, a β-glucan from Aureobasidium pullulans SM-2001, mitigates ovariectomy-induced osteoporosis in rats. *Exp Ther Med.* **12**, 1251-1262 (2016). doi: 10.3892/etm.2016.3485
9) Ma, Y.; Zeng, R.; Hu, Q.Q.; Yan, H.K.; Yang, L.X. et al. Preventive effects of *Polygonum orientale* L. on ovariectomy-induced osteoporosis in rats. *Climacteric.* **23**, 279-287 (2020). doi: 10.1080/13697137.2020.171462
10) Zhang, X.; Zhu, Y.; Zhang, C.; Liu, J.; Sun, T. et al. miR-542-3p prevents ovariectomy-induced osteoporosis in rats via targeting SFRP1. *J. Cell. Physiol.* **233**, 6798-6806 (2018). doi: 10.1002/jcp.26430
11) Tao, Z.-S.; Zhou, W.-S.; Wu, X.-J.; Zhang, X.; Wang, L. et al. Prevention of ovariectomy-induced osteoporosis in rats. *Z. Gerontol. Geriatr.* **52**, 139-147 (2019). doi: 10.1007/s00391-018-1376-x
12) Xie, W.; Han, Y.; Li, F.; Gu, X.; Su, D. et al. Neuropeptide Y1 receptor antagonist alters gut microbiota and alleviates the ovariectomy-induced osteoporosis in rats. *Calcif. Tissue Int.* **106**, 444-454 (2020). doi: 10.1007/s00223-019-00647-5
13) Chakuleska, L.; Michailova, R.; Shkondrov, A.; Manov, V.; Zlateva-Panayotova, N. et al. Bone protective effects of purified extract from Ruscus aculeatus on ovariectomy-induced osteoporosis in rats. *Food Chem Toxicol.* **132**, 110668 (2019). doi: 10.1016/j.fct.2019.110668
14) Du, M.-C.; Wu, B.; Ma, X.; Liu, Y.; Zhai, L.Q. et al. Protective effects of *Dihuang rougui* decoction on ovariectomy-induced osteoporosis in rats. *Trop. J. Pharm. Res.* **17**, 423-427 (2018).
15) Griffith, J.F.; Wang, Y.X.J.; Zhou, H.; Kwong, W.H.; Wong, W.T. et al. Reduced bone perfusion in osteoporosis: Likely causes in an ovariectomy rat model. *Radiology* **254**, 739-746 (2010). doi: 10.1148/radiol.09090608
16) Wang, Q.; Zhao, Y.; Sha, N.; Zhang, Y.; Li, C. et al. The systemic bone protective effects of Gushukang granules in ovariectomized mice by inhibiting osteoclastogenesis and stimulating osteoblastogenesis. *J. Pharmacol. Sci.* **136**, 155-164 (2018). doi: 10.1016/j.jphs.2018.01.007
17) Feng, J.; Liu, S.; Ma, S.; Zhao, J.; Zhang, W. et al. Protective effects of resveratrol on Postmenopausal osteoporosis: Regulation of SIRT1-NF-κB signaling pathway. *Acta Biochim. Biophys. Sin. (Shanghai).* **46**, 1024-1033 (2014). doi: 10.1093/abbs/gmu103
18) Long, L.; Han, X.; Ma, X.; Li, K.; Liu, L. et al. Protective effects of fisetin against myocardial ischemia/reperfusion injury. *Exp. Ther. Med.* **19**, 3177-3188 (2020). doi: 10.3892/etm.2020.8576
19) Chen, Y.P.; Sivalingam, K.; Shibu, M.A.; Peramaiyan, R.; Day, C.H. et al. Protective effect of Fisetin against angiotensin II-induced apotosis by activation of IGF-IR-P38-Akt signaling in H9c2 cells and spontaneous hypertension rats. *Phytomedicine* **57**, 1-8 (2019). doi: 10.1016/j.phymed.2018.09.179
20) Maher, P. Protective effects of fisetin and other berry flavonoids in Parkinson’s disease. *Food Funct.* **8**, 3033-3042 (2017). doi: 10.1039/c7fo00809k
21) Piao, M.J.; Kim, K.C.; Chae, S.; Keum, Y.S.; Kim, H.S.; Hyun, J.W. Protective effect of fisetin (3,7,3',4'-tetrahydroxyflavone) against γ-irradiation-induced oxidative stress and cell damage. *Biomed. Ther.* **21**, 210-215 (2013). doi: 10.4062/biomolther.2013.017
22) Li, Z.; Wang, Y.; Zhang, Y.; Wang, X.; Gao, B. et al. Pro-
Protective effects of fisetin on hepatic ischemia-reperfusion injury through alleviation of apoptosis and oxidative stress. Arch. Med. Res. 52, 163-173 (2021). doi:10.1016/j.arcmed.2020.10.009

23) Ahmad, S.; Khan, A.; Ali, W.; Jo, M.H.; Park, J. et al. Fisetin rescues the mice brains against D-galactose-induced oxidative stress, neuroinflammation and memory impairment. Front. Pharmacol. 12, 612078 (2021). doi:10.3389/fphar.2021.612078

24) Rajendran, M.; Ramachandran, R. Fisetin protects against rotenone-induced neurotoxicity through signaling pathway. Front. Biosci.-Elite 11, 20-28 (2019). doi:10.2741/E843

25) Watanabe, R.; Kurose, T.; Morishige, Y.; Fujimori, K. Protective effects of fisetin against 6-OHDA-induced apoptosis by activation of PI3K-Akt signaling in human neuroblastoma SH-SY5Y cells. Neurochem. Res. 43, 488-499 (2018). doi:10.1007/s11064-017-2445-z

26) Mustafa, R.A.; Alfky, N.A.A.; Hijazi, H.H.; Header, E.A.; Azzeh, F.S. Biological effect of calcium and Vitamin D dietary supplements against osteoporosis in ovariectomized rats. Prog. Nutr. 6, 143-149 (2018). doi:10.23751/pn.v20i1.5223

27) Fouda, A.-M.; Youssef, A.R. Antiosteoporotic activity of Salvadora persica sticks extract in an estrogen deficient model of osteoporosis. Osteoporos Sarcopenia 3(3), 132-137 (2017). doi:10.1016/j.jfos.2017.07.002

28) Wang, Y.G.; Jiang, L.B.; Gou, B. Protective effect of vanillic acid on ovariectomy-induced osteoporosis in rats. African J. Tradit. Complement. Altern. Med. 14(4), 31-38 (2017). doi:10.21010/ajtcam.v14i4.4

29) Sadat-Ali, M.; Al-Dakheel, D.A.; Al Mousa, S.A.; AlAnii, F.M.; Ebrahim, W.Y. et al. Stem-cell therapy for ovariectomy-induced osteoporosis in rats: A comparison of three treatment modalities. Stem Cells Cloning Adv. Appl. 12, 17-25 (2019). doi:10.2147/SCCAA.S204099

30) Zhang, X.; Xu, X.; Liu, X.; Mao, C.; Qin, A.; Lu, E. Bisbenoxacin blocks alveolar bone resorption in rats with ovariectomy-induced osteoporosis. Mol. Med. Rep. 17, 3232-3238 (2018). doi:10.3892/mmr.2017.8223

31) Liu, T.; Ding, S.; Yin, D.; Cuan, X.; Xie, C. et al. Pu-erh tea extract ameliorates ovariectomy-induced osteoporosis in rats and suppresses osteoclastogenesis in vitro. Front. Pharmacol. 8, 324 (2017). doi:10.3389/fphar.2017.00324

32) Rochel, N.; Wurtz, J.M.; Mitschner, A.; Klaholz, B.; Morris, D. The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. Mol. Cell 5, 173-179 (2000). doi:10.1016/S1097-2765(00)80413-X

33) Zhang, J.; Chalmers, M.J.; Stayrook, K.R.; Burris, L.L.; Garcia-Ordonez, R.D. et al. Hydrogen/deuterium exchange reveals distinct agonist/partial agonist receptor dynamics within Vitamin D receptor/retinoid X receptor heterodimer. Structure 18, 1332-1341 (2010). doi:10.1016/j.str.2010.07.007

34) Wang, X.; Chen, L.; Peng, W. Protective effects of resveratrol on osteoporosis via activation of the SIRT1-NF-κB signaling pathway in rats. Exp. Ther. Med. 14, 5032-5038 (2017). doi:10.3892/etm.2017.5147

35) Zhang, D.W.; Wang, Z.L.; Qi, W.; Zhao, G.Y. The effects of Cordyceps sinensis phytoestrogen on estrogen deficiency-induced osteoporosis in Ovariectomized rats. BMC Complement. Altern. Med. 14, 484 (2014). doi:10.1186/1472-6882-14-484

36) Ginaldi, L.; Di Benedetto, M.C.; De Martinis, M. Osteoporosis, inflammation and ageing. Immun. Ageing 2, 14 (2005). doi:10.1186/1742-4933-2-14

37) Mundy, G.R. Osteoporosis and inflammation. Nutr. Rev. 65(12 Pt 2), S147-S151 (2007). doi:10.1111/j.1753-4887.2007.tb00353.x

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