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ORIGINAL ARTICLE

Pila globosa Snail Extract Inhibits Osteoclast Differentiation via Downregulation of Nuclear Factor κB and Nuclear Factor of Activated T-Cells c1 Signaling Pathways

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ABSTRACT

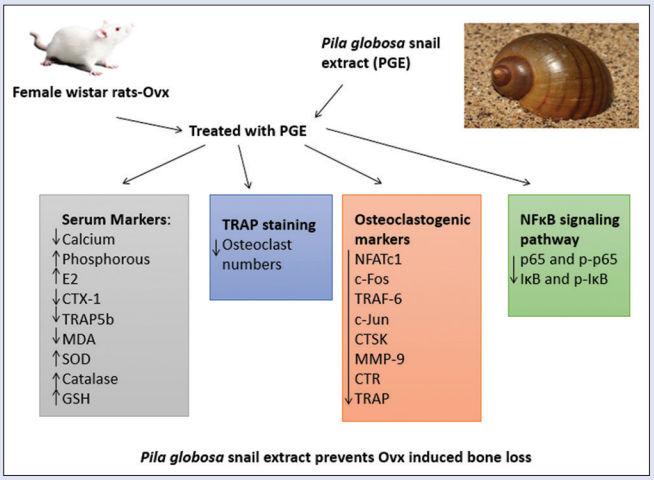
Background: Osteoporosis, a skeletal disease, that leads to increased fracture risk, features an enhanced osteoclast formation and bone resorption. Identification of agents that modulate aberrant osteoclast formation and function is important for the treatment of osteoporosis. **Objective:** The current study describes for the first time that *Pila globosa* snail extract inhibits osteoclastogenesis *in vitro* and thus suppresses bone loss in ovariectomy-induced rat model. **Materials and Methods:** Ovariectomized (Ovx) rats were treated with *P. globosa* snail extract and compared with sham, Ovx, and Ovx treated with zoledronate groups. Serum levels of C-terminal crosslinking telopeptides of type-1 collagen (CTX-1), TRAP5b, and antioxidant markers were determined. mRNA expressions of cathepsin K (CTSK), TRAP, calcitonin receptor (CTR), and matrix metalloproteinase 9 (MMP-9) were also assessed. Immunoblots of nuclear factor of activated T-cells c1 (NFATc1), c-Fos, TNF receptor-associated factor 6, c-Jun, and nuclear factor κB (NFκB) proteins were analyzed. **Results and Discussion:** *P. globosa* snail extract induced a decrease in the activation of NFκB, c-Fos, and NFATc1, which resulted in the downregulation of target genes, CTSK, TRAP, CTR, and MMP-9. *P. globosa* snail extract decreased the serum markers of bone resorption, C-terminal telopeptides of type-1 collagen (CTX-1), and tartrate-resistant acid phosphatase 5b (TRAP5b), reflecting the reduced number and activity of osteoclasts. Moreover, the results also suggested that the protective effect of *P. globosa* snail extract against osteoporosis is associated with the reduction of oxidative stress as evidenced by decreased malondialdehyde and increased serum antioxidant markers, superoxide dismutase, catalase, and glutathione. **Conclusion:** The upshot of the study suggests that the *P. globosa* snail extract represents a potential treatment option against osteolytic bone disease.

Key words: Osteoclastogenesis, osteoporosis, ovariectomy, oxidative stress, *Pila globosa*

SUMMARY

- Ovariectomized (Ovx) rats are treated with *Pila globosa* snail extract to determine the changes in the osteoclastic factors. This study reports that PG could decrease bone loss of the Ovx rats.

Abbreviations used: CCL_4: Carbon tetrachloride; CTR: Calcitonin receptor; CTSK: Cathepsin K; ELISA: Enzyme-linked immunosorbent assay; ERK: Extracellular regulated kinases; GSH: Glutathione; IκB: I kappa B; JNK: c-Jun N-terminal kinases; MAPK: Mitogen-activated protein kinase; MMP-9: Matrix metalloproteinase 9; OCN: Osteocalcin; Ovx: Ovariectomized; RANK: Receptor activator of nuclear factor κB; SOD: Superoxide dismutase; TRAP: Tartrate-resistant acid phosphatase.



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INTRODUCTION

Osteoporosis is a skeletal disorder characterized by decreased bone mineral density, deterioration of bone structure, and increased bone fracture risk (NIH Consensus Development Panel, 2001). India is the second most populated country in the world with approximately 10% of population (>100 million) over 50 years of age experience osteoporotic fractures.[1] In 2013, sources estimate that 50 million people in India are either osteoporotic or have low bone mass.[2]

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Increased bone resorption by osteoclasts is an indication of bone loss-associated diseases, such as osteoporosis, arthritis, osteomyelitis, periodontal bone loss, Paget's disease, and metastasis of tumors to bone.[3,4] Osteoclasts are bone-resorbing multinucleated giant cells differentiated from monocyte-macrophage lineage precursor cells.[5] The differentiation of osteoclast is induced by two key cytokines: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB (NFκB) ligand (RANKL). RANKL binds to its receptor RANK on the surface of osteoclast precursor cells and induces the recruitment of the adaptor protein TNF receptor-associated factor 6 (TRAF6). The formation of the RANKL-RANK-TRAF6 complex subsequently results in the activation of NFκB as well as three mitogen-activated protein kinases (MAPKs), including MAPK, extracellular regulated kinases, and c-Jun N-terminal kinases.[6] Ultimately, these signal transduction pathways lead to the expression and activation of transcription factors such as nuclear factor of activated T-cells c1 (NFATc1) and activator protein-1, both of which are involved in the expression of genes specific to osteoclasts.[7]

Oxidative stress is associated with the pathogenesis of osteoporosis.[8,9] There is a growing amount of evidence that oxidative stress induced by reactive oxygen species (ROS), during ovariectomy, can adversely affect bone homeostasis so that a pro-resorptive environment is favored.[10,11] Studies have shown that oxidative stress has an impact on osteoclast differentiation and functions.[12,13]

Thus, the mechanisms of most drugs currently used in the treatment of osteoporosis are focused on suppressing osteoclast-mediated bone resorption. Bisphosphonates have therefore been used in clinical practice, as therapeutic agents, in the management of osteoporosis and other bone diseases, such as hypercalcemia, metastatic bone disease, and Paget's disease.[14] Bisphosphonate exerts its effects by binding to hydroxyapatite in bone tissue, thereby inhibiting osteoclastic activity and inducing apoptosis of osteoclasts.[15] However, in recent years, adverse effects such as osteonecrosis of the jaws have been observed with the use of bisphosphonates such as zoledronate (Zol).[16,17]

Recently, natural products, such as honey, mussel, snake venom, curcumin, ginger, and black tea, are used for the treatment of osteoporosis and arthritis.[14,18,19] Snail extract has been ascribed with many therapeutic values and also have antioxidative properties. Snail *Helix aspersa* Müller extract is a good source of essential amino acid, fatty acid, vitamin, and minerals.[20] Giant African snail (*Archachatina marginata*) hemolymph has been proved to have antioxidant and hepatoprotective effects against carbon tetrachloride-induced hepatotoxicity in rats.[21] Slime protects against colon inflammation, is a good immune stimulant, and contains natural antioxidant molecules.[22] Górka *et al.*[23] showed that the eggs of snail *H. aspersa* possess antioxidant activity. Matusiewicz *et al.*[24] demonstrated the effect of snail *H. aspersa* Müller extract on the viability of human colorectal adenocarcinoma Caco-2 cells.

Viviparous bengalensis, a fresh water snail, a common Indian gastropod, is used as a traditional medicine against arthritis.[23] A previous study in an ovariectomized (Ovx) rat model of osteoporosis clearly established the importance of *V. bengalensis* snail extract as a possible nutraceutical which has therapeutic potential in preventing ovariectomy-induced osteoporotic changes.[25] However, the effect on osteoclast differentiation was not studied with *V. bengalensis* in osteoporosis animal model.

In the current study, we explored the effect of snail *Pila globosa* (PG) extract on osteoclast differentiation as well as its signaling pathways *in vitro* in Ovx rats. We hypothesized that the *P. globosa* snail extract reduces ovariectomy-induced bone loss by suppressing osteoclast differentiation and by modulating signaling molecules and osteoclast-specific genes. In the current study, we demonstrated that PG inhibits tartrate-resistant acid phosphatase (TRAPs), phenotypic marker of osteoclasts in Ovx rats *in vitro*, and suppresses the activation of NFκB, c-Fos, NFATc1, and osteoclast-specific genes including cathepsin K (CTSK), TRAP, calcitonin receptor (CTR), and matrix metalloproteinase 9 (MMP-9). Besides, in our study, PG reduces the serum level of Malondialdehyde(MDA) and increases antioxidant enzymes, superoxide dismutase (SOD), catalase, and glutathione (GSH). Thus, these results provided an evidence that *P. globosa* snail extract may be used as a natural anti-osteoclastogenic nutraceutical in the treatment of osteoporosis.

MATERIALS AND METHODS

Reagents

Recombinant human-soluble RANKL (sRANKL) and M-CSF were purchased from BioVision Inc., USA. All cell culture media and fetal bovine serum (FBS) were purchased from GIBCO, Grand Island, New York, USA. Antibodies against c-Fos, c-Jun, TRAF6, NFATc1, p65, p-p65, I kappa B (IκB), and p-IκB were purchased from Cell Signaling Technology (Danvers, MA, USA).

Preparation of *Pila globosa* extract

Fresh edible water snails *P. globosa* (PG) were collected from Chembarambakkam Lake, Chennai, Tamil Nadu, India. The snails *P. globosa* was identified by the Zoological Survey of India, Chennai. 0.1 g tissue was weighed, homogenized in 1 ml phosphate-buffered saline (0.01 M, pH 7.2), and centrifuged at 4000 rpm for 30 min at 4°C, and the supernatant was collected and stored at −20°C. The supernatant was used for treating Ovx rats.

Animals and experimental design

All of the animal procedures used were in strict accordance with the National Institute of Health Guidelines on the Care and Use of Laboratory Animals. The animals were allowed to acclimatize for a week before the experiment. Female Wistar rats (11 weeks of age, weight 230 ± 15 g) were housed at room temperature under a 12-h light/dark cycle and were weighed every week throughout the experimental period. Food and water were provided *ad libitum*. The animals were anesthetized using isoflurane (2%–2.5%) before surgery. Sham operations were performed by exteriorizing the ovaries and bilateral Ovx procedures were performed from the dorsal approach.

After surgery, rats were divided into four groups (n = 6/group); sham group, Ovx group, Ovx with Zol group (Ovx + Zol), and Ovx with *P. globosa* (Ovx + PG) snail extract group. The treatments were started 7 days after surgery. The Zol group received a single injection of Zol (20 μg/kg, sc; Novartis Pharma Schweiz AG) because reports had demonstrated that this minimum dose was required to provide long-term bone protection against the effects of OVX.[26,27] The *P. globosa* extract was administered orally (1 ml × 40 days). After 40 days of the treatment, the animals were euthanized, and at the end of the experiment, the blood samples were collected by cardiac puncture and the serum was separated for evaluating biochemical markers. The uterus was excised and the uterus weight was calculated. Urine was collected and urinary parameters were performed. Femurs were aseptically removed and bone marrow macrophages (BMMs) were isolated.

Determination of serum and urinary calcium and phosphorus

The concentrations of calcium and phosphorus in the serum and urine were measured using a commercial kit (QuantiChrom™ Bioassay Systems, Hayward, CA, USA).

Determination of serum estradiol (E2), TRAP5b, and CTX-1

Enzyme-linked immunosorbent assay (ELISA) assay was used to determine the level of serum E2. E2 kit was purchased from Tianjing Jiuding Biomedical Engineering Ltd. (Batch No. ESBL4287). The serum bone-specific resorption markers, tartrate-resistant acid phosphatase 5b (TRACP5b), and C-terminal telopeptide of type I collagen (CTX-1) were quantified in the serum samples using rat ELISA kits (Immunodiagnostic systems Inc., Stoughton, MA, USA). All experiments were performed according to the manufacturer's instructions, and the results were analyzed using a microplate reader (BioTek, USA).

Osteoclast differentiation and tartrate-resistant acid phosphatase staining

Bone marrow-derived adherent cells from femurs of female Wistar rats were seeded in 24-well plates at 2×10^6 cells/mL concentration in 0.2 mL of Dulbecco's modified Eagle's medium containing 10% FBS. Cells were cultured in the presence of RANKL (50 ng/mL) and M-CSF (30 ng/mL) for 14 days. The cells were re-fed every alternative day with fresh medium. After 14 days, cultured cells were fixed and stained for TRAP, an osteoclast marker protein. TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts.

Western blot analysis

Cells were lysed in RIPA lysis buffer (20 mM Tris–HCl [pH 7.5], 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM phenyl methyl sulfonyl fluoride, and 1x protease inhibitor cocktail). Protein concentrations were measured using BCA method (Pierce, USA). An equal amount of protein was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane (Whatman, United Kingdom). After blocking with 5% skim milk in Tris-buffered saline and Tween 20 (TBS-T buffer [0.1 M], Tris-HCl [pH 7.4], 0.9% NaCl, 0.05% Tween-20), the membranes were incubated with diluted primary antibodies against c-Fos, c-Jun, NFATc1, TRAF6, p65, p-p65, IκB, and p-IκB overnight at 4°C. After washing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody in 5% skim milk for 1 h at room temperature. After washing with TBS-T for 1 h, target proteins were visualized using ECL Plus kit (Thermo Scientific). Quantification of blots was done by densitometry analysis using Image Lab™ software (Bio-Rad, Hercules, CA, USA).

Isolation of total RNA and reverse transcription polymerase chain reaction

Total RNA was extracted with TriZol Reagent (Invitrogen), and complementary DNA (I Script cDNA Synthesis Kit, Invitrogen) was synthesized according to the manufacturer's instructions. The primer sequence for polymerase chain reaction (PCR) and the reaction conditions used in this study are listed in Table 1.

Measurement of serum malondialdehyde and superoxide dismutase, catalase, and glutathione

The serum levels of MDA, SOD, catalase, and GSH were estimated according to the manufacturer's instructions (Cayman Chemical Company, USA) and absorbance was also read using a microplate reader (BioTek, USA).

Statistical analysis

The data are presented as mean ± standard error of the mean and analyzed statistically by GraphPad Prism 7 version (GraphPad Statistical Software Inc., San Diego, CA, USA). The data were subjected to one-way ANOVA, followed by the *post hoc* (Tukey) test. $P < 0.05$ was considered statistically significant.

RESULTS

Body weight and uterus weight

The body weights were significantly [$P < 0.001$, Table 2] higher in the Ovx group as compared with the sham, Ovx + Zol, and Ovx + PG groups, while the uterus weight was significantly [$P < 0.001$, Table 2] lower in the Ovx group compared with the Ovx + PG and Ovx + Zol groups. The body weights decreased significantly [$P < 0.001$, Table 2] in the rats treated with PG and Zol groups compared with those in the Ovx-only group.

Serum calcium, phosphorus and urinary calcium, phosphorus

When comparing the Ovx group to the sham group, the levels of serum calcium and phosphorus were significantly lower [$P < 0.001$, Table 3]; however, the levels of urinary calcium and phosphorus were significantly higher. The levels of serum calcium and phosphorus tended to increase in the rats that treated with PG snail extract and Zol. The rats treated with PG extract exhibited decreased levels of urinary calcium, phosphorus, compared with Ovx group [Table 3].

Serum estradiol and bone resorption markers

Ovariectomy caused a significant increases in the serum CTx and TRAP5b levels [Table 4]. Treatment with PG or Zol suppressed the increases in serum CTx and TRAP5b levels in the Ovx rats. The levels of CTx and TRAP5b in the Ovx rats were 65.17% and 69.8%, respectively; these levels were higher than those in the sham-operated rats. PG treatment reduced the CTx level by 127.4% and the osteocalcin level by 86.8% compared to OVX rats.

Pila globosa snail extract attenuates osteoclasts formation

As shown in Figure 1, the number of TRAP-positive cells was increased in OVX-induced rats *in vitro* ($P < 0.001$). Cells treated with snail extract and Zol exhibited a significant [$P < 0.001$, Figure 1] decrease in number of TRAP-positive multinucleated cells, when compared to OVX rats. These results suggest that PG snail extract inhibits osteoclastogenesis.

Pila globosa snail extract inhibits TNF receptor-associated factor 6, c-Fos, nuclear factor of activated T-cells c1, and c-Jun expression

NFATc1/c-Fos/c-Jun represents a signaling pathway that is critical for osteoclasts differentiation and function. The OVX-induced increase in TRAF6, c-Fos, NFATc1, and c-Jun protein levels was significantly attenuated by snail extract and Zol treatment [Figure 2a and b].

Pila globosa snail extract inhibits activation of nuclear factor κB signaling in bone marrow macrophages

NFκB is one of the key transcription factors activated by the osteoclast differentiation factor RANKL. As shown in Figure 3a and b, the OVX-induced phosphorylation of IκB and the phosphorylation of p65 were significantly suppressed by snail extract. From these data, it was confirmed that PG snail extract and Zol inhibit osteoclastogenesis by inhibiting the NFκB activation.

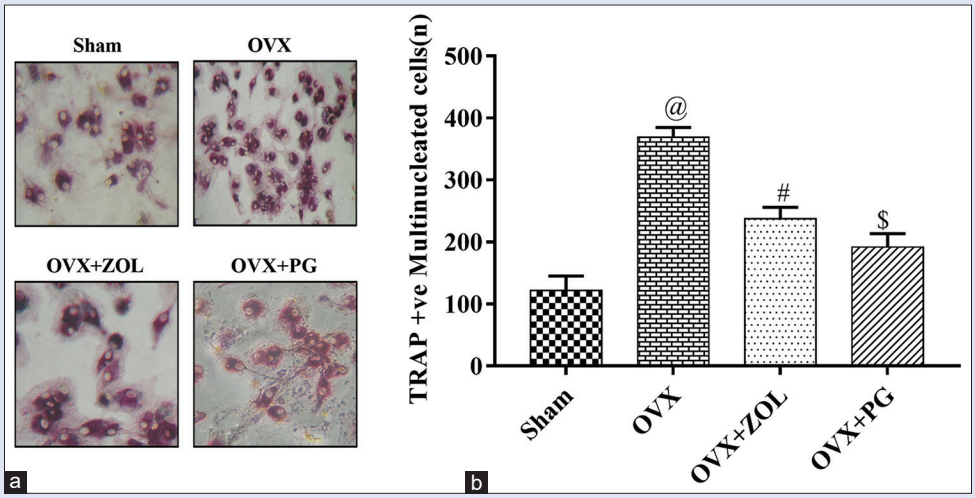

Figure 1: Effects of snail extract on osteoclastogenesis of bone marrow macrophages. Cells were incubated receptor activator of nuclear factor κB ligand and colony-stimulating factor and cultured for 14 days. Then, the cells were stained for tartrate-resistant acid phosphatase assay. (a) Representative images of tartrate-resistant acid phosphatase-positive cells, (b) relative number of tartrate-resistant acid phosphatase-positive multinucleated osteoclasts. All data are presented by mean ± standard deviation. [@]Significant difference compared between Sham versus OVX ($P < 0.001$), [#]Significant difference compared OVX versus OVX + ZOL ($P < 0.001$), [$]Significant difference compared OVX versus OVX + PG ($P < 0.001$). OVX: Ovariectomized; ZOL: Zoledronate; PG: *Pila globosa*

Table 1: List of primers

Target genes	Primers sequence	Annealing Tm (°C)	PCR cycle	Product size (bp)
CTSK	5′- CCGCAGTAATGACACCCTTT -3′ 5′- AAGGCATTGGTCATGTAGCC -3′	60	30	258
CTR	5′- ACTGCTGGCTGAGTGTGGAAA -3′ 5′- GAAGCAGTAGATGGTCGCAAC -3′	58	35	317
MMP-9	5′- TTGACAGCGACAAGAAGTGG -3′ 5′- CCCTCAGTGAAGCGGTACAT -3′	60	40	145
TRAP	5′- CCAATGCCAAAGAGATCGCC -3′ 5′- TCTGTGCAGAGACGTTGCCAAG -3′	55	40	216
β-actin	5′-TCACCCACACTGTGCCCATCTACGA-3′ 5′- CAGCGGAACCGCTCATTGCCAATGG-3′	64	30	295

PCR: Polymerase chain reaction; MMP-9: Matrix metalloproteinase 9; TRAP: Tartarate-resistant acid phosphatase; Tm: Melting temperature; CTSK: Cathepsin K; CTR: Calcitonin receptor

Table 2: Effects of ovariectomized and *Pila globosa* treatment on body and uterus weight (g)

Group	Body weight (g)	Uterus weight (g)
Sham	221.70±9.86	0.66±0.09
OVX	305±5.64[@]	0.412±0.03[@]
OVX + ZOL	256.66±6.58[#]	0.84±0.08[#]
OVX + PG	268.658±7.93[$]	0.645±0.06[$]

Data are presented as the mean±SD. [@]Significant difference compared between Sham versus OVX ($P<0.001$); [#]Significant difference compared OVX versus OVX + ZOL ($P<0.001$); [$]Significant difference compared OVX versus OVX + PG ($P<0.001$). OVX: Ovariectomized; ZOL: Zoledronate; PG: *Pila globosa*; SD: Standard deviation

Pila globosa snail extract suppresses the expression of osteoclast maker genes

To study further the effects of snail extract on osteoclast differentiation, reverse transcription (RT)-PCR was used to analyze the mRNA levels of four osteoclast-specific genes (TRAP, CTSK, CTR, and MMP-9) in BMM cells. RT-PCR analysis as shown in Figure 4 demonstrated that the expressions of CTSK (1.42-fold, $P < 0.001$), CTR (1.34-fold, $P < 0.001$), MMP-9 (1.64-fold, $P < 0.001$), and TRAP (1.61-fold, $P < 0.001$) were higher in the OVX rats than in the sham-operated rats. Snail extract treatment led to 0.58-fold ($P < 0.001$) decrease in CTSK, 0.28-fold ($P < 0.001$) decrease in CTR, 0.80-fold ($P < 0.001$) decrease in MMP-9, and 0.90-fold ($P < 0.001$) decrease in TRAP expression. Zol treatment led to 0.89-fold ($P < 0.001$) decrease in CTSK, 0.60-fold ($P < 0.001$) decrease in CTR, 0.88-fold ($P < 0.001$) decrease in MMP-9, and 0.73-fold ($P < 0.001$) decrease in TRAP expression.

Effect of *Pila globosa* snail extract on serum biochemical markers of oxidative stress

As shown in Table 5, in contrast to the sham-operated group, the OVX group exhibited enhanced MDA and reduced SOD, catalase, and GSH levels. However, PG extract treatment significantly reduced the serum MDA and elevated SOD, catalase, and GSH levels. Snail extract treatment showed 53% increase in SOD level, 64.6% increase in catalase level, and 68.2% increase in GSH level; whereas Zol increases 53.9% in SOD, 67.8% in catalase level, and 70.5% in GSH, when compared to OVX rats. These results demonstrated that the beneficial effects of snail extract may also be associated with its antioxidant activity.

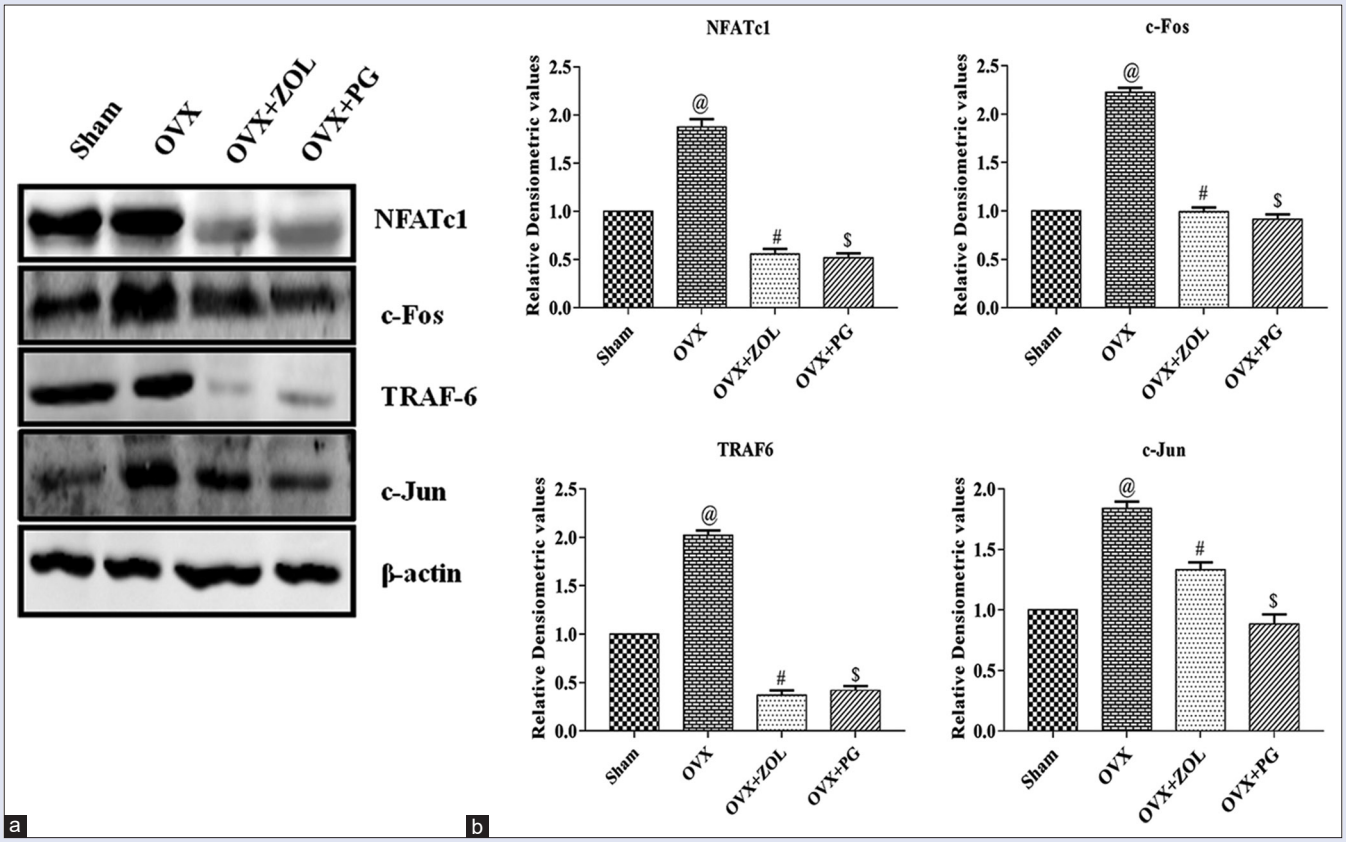

Figure 2: Snail extracts suppressed the activation of nuclear factor of activated T-cells c1/c-Fos pathway. Expressions of nuclear factor of activated T-cells c1, c-Fos, TRAF6, and c-Jun proteins were analyzed by western blot. (a) Representative blot image. (b) Relative densitometric analysis in histograms. Results were presented by mean ± standard error of the mean. [@]Significant difference compared between Sham versus OVX ($P < 0.001$), [#]Significant difference compared OVX versus OVX + ZOL ($P < 0.001$), [$]Significant difference compared OVX versus OVX + PG ($P < 0.001$). OVX: Ovariectomized; ZOL: Zoledronate; PG: *Pila globosa*

Table 3: Effects of ovariectomized and *Pila globosa* treatment on levels of serum calcium, phosphorus and urinary calcium, phosphorus, creatinine, and DPD crosslinks

Group	Serum calcium (mmol/l)	Serum phosphorus (mmol/l)	Urinary calcium (mmol/l)	Urinary phosphorus (mmol/l)	Urinary creatinine (mmol/l)	Urinary DPD (nmol/mmol)
Sham	7.21±0.65	6.26±0.23	4.57±0.35	8.65±0.65	4.25±0.99	8.30±0.42
OVX	10.25±0.80[@]	4.72±0.18[@]	8.15±0.60[@]	11.24±0.90[@]	7.47±0.49[@]	14.56±0.91*
OVX + ZOL	7.64±0.97[#]	5.14±0.15**	6.15±0.50[#]	8.40±0.62[#]	5.31±0.40[#]	10.31±0.46[#]
OVX + PG	7.38±0.83[$]	5.23±0.23**	5.27±0.61[$]	8.13±0.91[$]	4.34±0.50[$]	9.44±0.35[$]

Data are presented as the mean±SD. [@]Significant difference compared between Sham versus OVX ($P<0.001$); [#]Significant difference compared OVX versus OVX + ZOL ($P<0.001$); [$]Significant difference compared OVX versus OVX + PG ($P<0.001$); *Significantly different ($P<0.05$) from OVX; **Significantly different ($P<0.01$) from OVX. OVX: Ovariectomized; ZOL: Zoledronate; PG: *Pila globosa*; DPD: Deoxypyridinoline

Table 4: Effect of *Pila globosa* on serum parameters of bone turn over markers

Group	E2 (pg/ml)	Serum CTX-1 (ng/ml)	Serum TRAP5b (U/L)
Sham	43.11±12.49	11.21±0.9	6.5±0.78
OVX	5.38±0.89	17.2±0.6[@]	9.3±1.3[@]
OVX + ZOL	37.88±11.61	12.3±1.5[#]	7.6±0.91*
OVX + PG	34.79±8.23	13.5±1.1[$]	6.9±0.86**

Data are presented as the mean±SD. [@]Significant difference compared between Sham versus OVX ($P<0.001$); [#]Significant difference compared OVX versus OVX + ZOL ($P<0.001$); [$]Significant difference compared OVX versus OVX + PG ($P<0.001$); *Significantly different ($P<0.05$) from OVX; **Significantly different ($P<0.01$) from OVX. OVX: Ovariectomized; ZOL: Zoledronate; PG: *Pila globosa*; SD: Standard deviation; TRAP5b: Tartrate-resistant acid phosphatase 5b

DISCUSSION

In this study, we have demonstrated that *P. globosa* snail extract, a nutraceutical, is capable of inhibiting osteoclast formation and inhibiting osteoclast marker gene expression through blocking NFκB and NFATC1 signaling pathways. This indicates that *P. globosa* snail extract could be utilized as a therapy to prevent bone resorption in ovariectomy-induced osteoporosis.

After ovariectomy, serum estradiol level in the OVX group was significantly lower than in the sham group [Table 2], which confirms the ovarian deficiency in the Ovx model. In addition, body weights were greater in the Ovx group than in the sham group and an atrophy of the uterus was also observed in Ovx rats [Table 2]. Our results are comparable to those of previous studies that have used Ovx rat

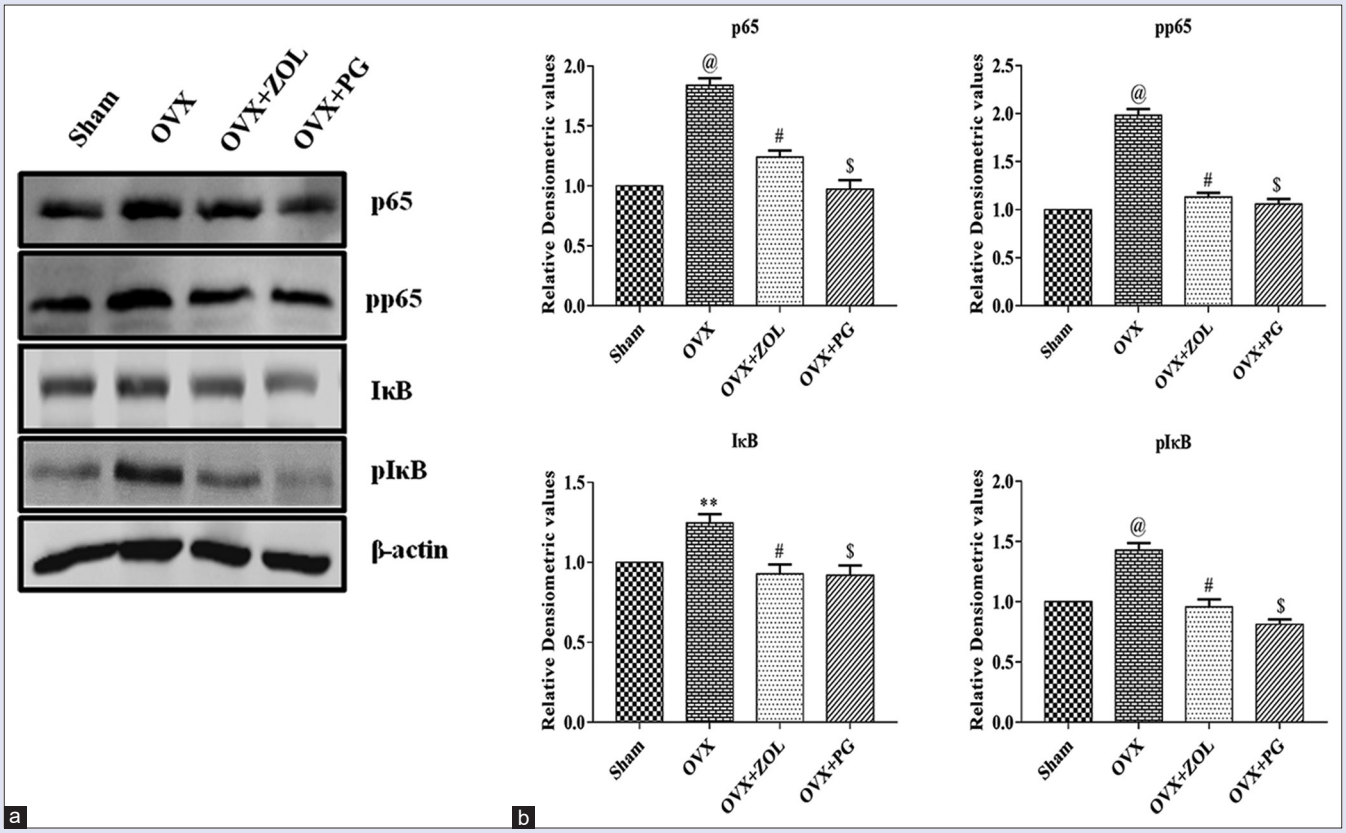

Figure 3: Snail extract inhibits receptor activator of nuclear factor κB ligand stimulated activation of nuclear factor κB signaling pathway in bone marrow macrophages. Total protein extracts were analyzed by western blot. (a) Representative blot image. (b) Relative densitometric analysis in histograms. Results were presented by mean ± standard error of the mean. @Significant difference compared between Sham versus OVX ($P < 0.001$), #Significant difference compared OVX versus OVX + ZOL ($P < 0.001$), $Significant difference compared OVX versus OVX + PG ($P < 0.001$). **Significantly different compared to Sham versus OVX ($P < 0.01$). OVX: Ovariectomized; ZOL: Zoledronate; PG: *Pila globosa*

Table 5: Effect of *Pila globosa* in serum malondialdehyde, superoxide dismutase, catalase and glutathione activities

Group	MDA (nM/ml)	SOD (U/ml)	Catalase (nM/min/ml)	GSH (U/ml)
Sham	3.8±0.31	331.6±5.29	72.4±0.54	77.5±0.62
OVX	13.44±1.41@	173.3±4.17@	42.3±0.38@	48.8±0.41@
OVX + ZOL	5.26±0.43#	321.2±8.13#	62.3±0.46#	69.2±0.49#
OVX + PG	6.76±0.56$	324.3±5.5$	65.4±0.55$	71.5±0.49$

Data are presented as the mean±SD. @Significant difference compared between Sham versus OVX ($P<0.001$); #Significant difference compared OVX versus OVX + ZOL ($P<0.001$); $Significant difference compared OVX versus OVX + PG ($P<0.001$). OVX: Ovariectomized; ZOL: Zoledronate; PG: *Pila globosa*; SD: Standard deviation; MDA: Malondialdehyde; SOD: Superoxide dismutase; GSH: Glutathione

model.[25,28,29] The increase in body weight and atrophy of the uterus in Ovx rats are ascribed to estrogen deficiency,[30] indicating ovariectomy. However, in the treated groups, a significant decline in body weight and uterus weight was noted, indicating that the *P. globosa* snail extract and Zol could have improved the osteoporotic condition of the rats that underwent ovariectomy.

Results from this study also showed that the serum calcium and phosphorus levels of Ovx rats were significantly lower than those of the sham-operated group [Table 3], reflecting the degree of bone loss. This result is also in accordance with the findings of Zhou *et al*. and Wu *et al.*,[31,32] who also reported lower serum level of calcium and phosphorus in Ovx mice in comparison to sham group. It has been demonstrated that estrogen decrease in Ovx rats suppresses intestinal absorption of calcium, resulting in reduced serum calcium level.[32] We also showed that *P. globosa* snail extract and Zol treatment increased the serum levels of calcium and phosphorus [Table 3]. Sarkar *et al.*[25] reported that fresh water snail *V. bengalensis* flesh extract decreased serum calcium, phosphorus, and creatinine levels in Ovx rats.

Ovx rats, in the present study, had an increased loss of urinary calcium and Phosphorus levels compared to the sham group, indicating an increase in net bone resorption [Table 3]. Rudzki *et al.*[33] showed that an increase in loss of calcium, phosphorus through excretion in urine indicates bone loss in the Ovx rats. However, these responses were significantly lowered in Ovx rats on receiving *P. globosa* snail extract and Zol treatment, attributing to decreased bone resorption. This result suggests that, compared with Zol, retention of these bone minerals and creatinine could be better achieved by *P. globosa* snail extract.

Bone resorption in Ovx rats can be assessed by several biochemical markers, e.g., serum C-terminal telopeptides of type-1 collagen (CTX-1) and TRAP-5b. CTx, a degradation product of bone collagen, is a reliable marker of the resorption activity of osteoclasts. In contrast, TRAP may reflect different aspects of osteoclast function and degradation of noncollagenous proteins.[34] In the present study, Ovx animals were found to have higher serum CTX-1 and TRAP5b levels than sham control [$P < 0.001$, Table 4], indicating bone deterioration and loss. Kim *et al.* and Yoon *et al.*[35,36] reported an increased CTX-1 level in OVX rats. Miyauchi *et al.*[37] suggested that the estrogen depletion could

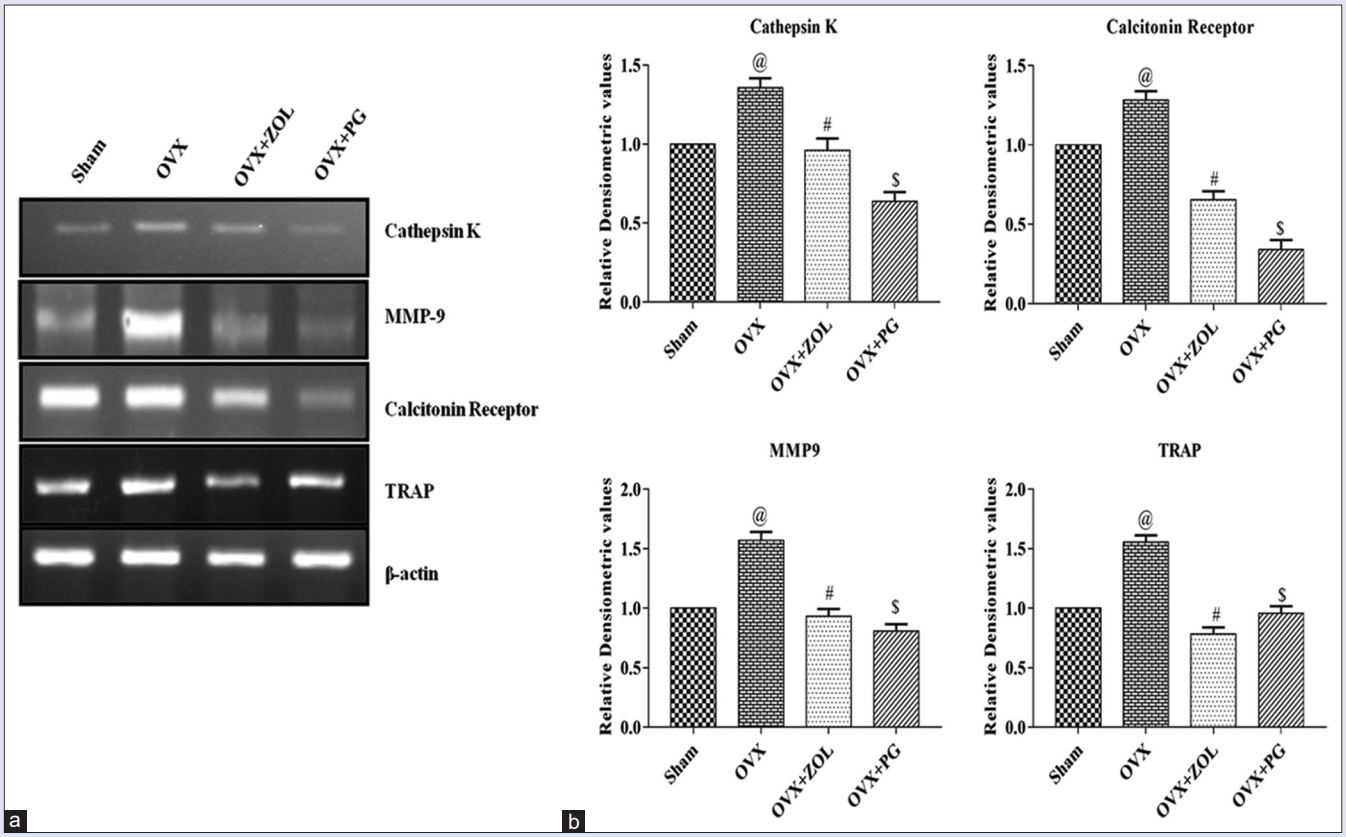

Figure 4: Snail extract suppressed receptor activator of nuclear factor κB ligand stimulated expression of osteoclast specific genes. mRNA expression of Cathepsin K, Calcitonin Receptor, matrix metalloproteinase 9 and tartrate-resistant acid phosphatase were analyzed by reverse transcription polymerase chain reaction. (a) Representative reverse transcription polymerase chain reaction gel image. (b) Relative densitometric analysis in histograms. Results were presented by mean ± standard error of the mean. @Significant difference compared between Sham versus OVX ($P < 0.001$), #Significant difference compared OVX versus OVX + ZOL ($P < 0.001$), $Significant difference compared OVX versus OVX + PG ($P < 0.001$). OVX: Ovariectomized; ZOL: Zoledronate; PG: *Pila globosa*

trigger the increase of CTx in the serum. Moreover, serum TRAP-5b, an osteoclast-derived enzyme, reflects the number and activity of osteoclasts on bone surface.[38] Hence, from the results, it is evident that there is a significant increase in osteoclastic activity, leading to greater resorption of bone. However, treatment with PG and Zol decreases serum CTx and TRAP5b elevation in Ovx rats, thus indicating the reduction in osteoclast number.

Osteoclasts differentiate from monocyte/macrophage lineage hematopoietic precursor cells at different stages of proliferation, migration, fusion, and activation.[39] Increased osteoclast numbers are implicated in the development of bone loss-associated diseases, such as osteoporosis.[40]

The study of TRAP-positive cell formation and activity is a well-known method of determining osteoclast formation and function.[41] The present result showed an increased proportion of mononuclear cells positive for TRAP, a marker of osteoclasts lineage, in cells derived from Ovx rats [$P < 0.001$, Figure 1] than sham group *in vitro*. The result agrees with the studies of D'Amelio *et al.*,[42] showing higher number of osteoclasts in Ovx rats. This is also in accordance with previous study which has shown an increased TRAP staining in Ovx mice as an indicative of enhanced osteoclast numbers.[43] The result implies that estrogen depletion would result in the priming of large number of osteoclast precursors to differentiate into mature osteoclasts.[43,44] Moreover, OCs in the Ovx group were larger than sham group. Trebec *et al.*[45] demonstrated that larger OCs are more active at bone resorption sites than smaller cells, suggesting for excessive bone loss. In the present study, we noted that PG and Zol inhibited osteoclast formation. Abe *et al.*[46] investigated that treatment with Zol directly inhibited RANKL-stimulated osteoclast differentiation and fusion in RAW 264.7 cells.

In particular, key transcription factors, including NFκB, c-Fos, and NFATc-1, are known to play an essential role in osteoclastogenesis.[47] Previous study showed that p65-deficient cells failed to differentiate into osteoclasts due to impaired survival, suggesting a critical role of the NFκB signaling pathway in osteoclast differentiation.[48] We showed that PG suppressed the osteoclast differentiation through inhibition of Iκβ phosphorylation [Figure 3] in the NFκB signaling pathway. NFATc1 is a master transcription factor involved in the terminal differentiation of osteoclasts via upregulation of various osteoclast-specific genes.[49] Importantly, c-Fos-deficient mice develop osteoporosis due to impaired osteoclast formation.[50] Therefore, suppression of NFATc1 and c-Fos by Zol and *P. globosa* snail extract treatment [Figure 2] indicates the inhibition of osteoclast differentiation.

NFATc1 regulates the expression of genes involved in the osteoclast differentiation and function, such as TRAP, CTSK, CTR, and MMP-9.[51] CTSK is a cysteine lysosomal protease that is selectively and highly expressed by bone-resorbing osteoclasts and has been proposed to play a key role in bone matrix and thus bone resorption.[52] The expression of CTR signals an important stage of osteoclast development and regulated by NFATc1.[53] The present data support the hypothesis that the higher mRNA expression of CTSK, TRAP, CTR, and MMP-9

observed in the Ovx group [Figure 4] might indicate that an increased number of mature osteoclasts strives to resorb bone. These findings are consistent with the studies of Wang *et al.*,[54] who reported an increased activity of CTSK and CTR in Ovx mice. Kim *et al.*[55] found the increased mRNA expression of CTSK and CTR in RAW 264.7 cells.

Therefore, to determine the mechanism by which PG suppresses osteoclast differentiation, we confirmed the effect of PG on the mRNA levels of NFATc1, TRAP and CTSK, CTR. PG extract not only suppressed the NFATc1 but also suppressed the downstream effectors such as TRAP, CTSK, CTR, and MMP-9 and this suppression corresponded to PG treatment induced decrease in the number of osteoclasts. Zol inhibited the mRNA expression of TRAP, which is an osteoclast-specific marker.[46] In the present study, serum MDA level was increased significantly in Ovx group [$P < 0.001$, Figure 3] than those of in the sham control [$P < 0.01$, Figure 3], which is in line with the results of Ha *et al.*[56] Moreover, the present result regarding MDA levels in Ovx group is in agreement with Yalin *et al.*,[57] who reported an increased MDA concentration in Ovx rats. It can be suggested that ROS generated during ovariectomy could accelerate the activity of lipid peroxidation and thus the increased level of MDA. Estrogen deficiency has been shown to be associated with an increase in the production of lipid peroxides and a deficient antioxidant defense, resulting in the pathogenesis of osteoporosis.[58] Osteoclastic activity enhanced in bone disorders may be responsible for increased production of ROS in superoxide forms, which is evident by increased levels of serum MDA.[59]

In the current study, the serum levels of the antioxidant enzymes, SOD, CAT, and GSH, were significantly lower in OVX group [$P < 0.001$, Figure 3] as compared to sham [$P < 0.01$, Figure 3]. In rat femurs, ovariectomy results in oxidative stress and decreases the capacity of antioxidant defense mechanisms.[60] NFκB and NFATc1 activation observed in OVX group can also be correlated to oxidative stress response. It has been reported that anti-osteoclast effect via ROS inhibition is often accompanied by suppressing the gene expression of NFATc1.[61]

The present results demonstrated that PG snail extract and Zol decreased MDA and improved serum antioxidative markers, including reduced SOD, catalase, and GSH [Table 5]. Gold nanoparticles suppressed the NFκB activation and reduced the ROS level.[62] Rutin acted as an antioxidant to inhibit osteoclastogenesis via decreased the NFκB activation.[63] It could be concluded that oxidative stress and decreased antioxidant defenses may have an important role in the pathogenesis of ovariectomy-induced osteoporosis.

CONCLUSION

PG snail extract attenuated OVX-induced bone loss through multiple mechanisms; First, snail extract directly inhibited osteoclast formation, which might be the most important mechanism; second, by regulating c-Fos/NFATc1/NFκB signaling and suppressing downstream effectors such as CTSK, MMP-9, CTR, and TRAP which might inhibit osteoclastogenesis; third, snail extract was shown to reduce serum concentrations of osteolytic markers including CTX-1 and TRAP5b which might also lead to suppressed osteoclastogenesis; fourth, protection by snail extract against osteoporosis is partly associated with a reduction in oxidative stress.

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Conflicts of interest

There are no conflicts of interest.

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