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Research Article
Soybean Sprouts Inhibit Bone Turnover in Ovariectomized Rats

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Abstract

Background and Objective: Soybeans are known for their positive influence on the prevention of osteoporosis in postmenopausal women. Soybean sprouts produce bioactive compounds that are better than soybeans. This study aimed to compare the potential of soybean sprouts with soybean and ethinylestradiol on the changes of biomarkers of bone turnover activity in ovariectomized rats (OVX). **Materials and Methods:** Twenty-five female Sprague-Dawley rats aged 2 months were placed into 5 groups: (i) normal control (without OVX) (N-C); (ii) OVX control (OVX-C); (iii) OVX+ethinylestradiol (30 μg kg^{-1} b.wt.,/day, orally) (OVX-E); (iv) OVX+soybean flour (based on a dose 10 μg g^{-1} b.wt.,/day of isoflavones, orally) (OVX-S); (v) OVX+soybean sprout flour (based on a dose 10 μg g^{-1} b.wt.,/day of isoflavones, orally) (OVX-SS). All groups were treated for 6 weeks and all rats were fed an AIN-93M-based diet. Blood samples were collected before and after treatment for analysis of serum biomarkers of bone turnover and estradiol hormone. Data were analyzed using one-way ANOVA, followed by Duncan's Multiple-Range (DMR) test. **Results:** The increase of osteocalcin (OC) and beta-crosslaps (βCTx) in the serum of the OVX-SS group was lower than in the OVX-S group and was the same as the OVX-E group. The OVX-C group experienced the highest increase in OC and βCTx. All groups of OVX rats also experienced a significant decline in estradiol hormone. There was no difference in the decrease in serum estradiol in the OVX-S and OVX-SS groups. **Conclusion:** The results of the study show that soybean sprout flour consumption provides better inhibition of bone turnover activity than soybeans in ovariectomized rats. The potential of soy and soybean sprouts in estradiol hormone recovery on ovariectomized rats is not different.

Key words: Ovariectomized rats, sprouts, soybean, bone turnover, postmenopausal women

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Osteoporosis is often called a silent disease because it does not show symptoms until bones are fragile and fractures occur[1]. Women are at higher risk for osteoporosis than men, as this disease is associated with the decreased production of estrogen[2,3]. Estrogen plays an important role in the maintenance of bone density by regulating the formation and resorption of bone by osteoblast and osteoclast cells[4]. Bone turnover activity can be measured via serum biochemical markers, such as osteocalcin (OC) and beta-crosslaps (βCTx)[5]. Estrogen deficiency in postmenopausal women is associated with increased bone turnover activity. High bone turnover activity leads to bone loss, making bones fragile and susceptible to fractures[1].

Estrogen deficiency can be improved through Hormonal Replacement Therapy (HRT). HRT is known to cause undesirable side effects, such as increased risk of heart disease, endometrial cancer and ovarian disease[6,7].

One alternative to HRT is the use of phytoestrogens, especially isoflavones[8]. Several studies show the positive role of isoflavones on the prevention of bone loss[9,10]. Soybeans are natural source of isoflavones. Soybean consumption could be encouraged as an alternative to estradiol treatment for a safer and more natural approach to HRT. Park *et al.*[11] and Alekel *et al.*[12] have shown that the consumption of soybeans and soybean extract, both in ovariectomized rats and postmenopausal women, could improve the activity of biochemical markers of bone formation and increase bone mass. However, soybeans contain compounds that interfere with the absorption of nutrients, such as trypsin inhibitors, acid phytat, α-galactosidase, tannin, hemaglutinin and oxalate[13,14]. Using simple technology to affect the germination of soybeans can improve nutritional components, such as protein, free amino acids, α-tocopherol, vitamin C, fiber, etc. and increase the bioactive compounds, such as polyphenols and isoflavones[15,16]. Artificial germination is also able to reduce the anti-nutritional components of soybeans[15,17].

The bioactive compounds of soybean sprouts are better than those of soybeans. Soybean sprouts have better potential in the prevention of bone loss in postmenopausal women and in the rat model as detected through biochemical markers of osteoblast and osteoclast cell activity and by an increase in the estrogen hormone expressed by levels of OC, βCTx and serum estradiol. The study aimed to compare the potential of soybean sprouts with soybeans and ethinylestradiol on the changes in biochemical markers of bone turnover activity in ovariectomized rats (OVX), or postmenopausal model rats.

MATERIALS AND METHODS

Materials: Soybean seed varietal Anjasmoro was obtained from Balai Penelitian Kacang-kacangan dan Umbi-umbian Kendalpayak Malang, Jawa Timur Indonesia. Ethinylestradiol 0.05 mg/tablet (Lynoral) was obtained from PT Sydna Farma, Jakarta Indonesia. Osteocalcin (OC) and beta-Crosslaps (βCTx) Enzyme-linked Immuno Sorbent Assay (ELISA) kits were obtained from Cloud-Clone Corp (USA), while estradiol ELISA kits were obtained from DRG (Germany).

Preparation of soybeans and soybean sprout flour: The germination process was initiated by soaking the soybeans in a 2% NaCl solution for 8 h. Germination continued for 36 h in a dark environment (modification of Rusydi and Azrina)[17]. Soybean sprouts were then dried using a cabinet dryer for 12 h at a temperature of ±50°C. Both the soybean flour and the soybean sprout flour were prepared using a disk mill and sieve (mesh size 80). The flour was then packed in polyethylene bags for later use.

Animals and diets: This study obtained ethical clearance from the Commission on Ethical Clearance LPPT UGM, No: 206/KEC-LPPT/XII/014. Twenty-five female Sprague Dawley rats aged 2 months were obtained from the Integrated Research and Testing Laboratory (LPPT) at the Universitas Gadjah Mada (UGM) for the experiement. Rats were raised in individual cages in an air-conditioned room at a temperature of 26-29°C; humidity: 60-70%, with a 12 h cycle of light and dark. After adapting to the environment for the first week, 20 rats were ovariectomized (OVX), while five other rats were dissected without ovarian retrieval as the normal control group. The rats were anesthetized using ketamine (10%) and xylazine (2%), given as an intramuscular injection prior to surgery[18]. All rats were fed -93 M diet[19] through the 1-week recovery period. Next, the rats were randomly divided into five groups: (i) normal control (without OVX) (N-C); (ii) OVX control (OVX-C); (iii) OVX+ethinylestradiol (OVX-E); (iv) OVX+soybean flour (OVX-S); (v) OVX+soybean sprout flour (OVX-SS). All rats were fed a base diet of AIN-93 M. All treatments were given orally for 6 weeks, with the amount of soybean flour and soybean sprout flour given based on a dose 10 μg g^{-1} b.wt.,/day of isoflavones. Treated with ethinylestradiol at 30 μg kg^{-1} b.wt.,/day. During the experiment, the weight of each rat was checked every 2 days.

Sample collection: Blood samples were collected before and after treatment to analyze the biochemical serum. After an

overnight fast, blood samples were taken from the rats' orbital sinus under anesthesia with an intramuscular injection of ketamine (10%) and xylazine (2%). Blood was collected in microtubes and centrifuged at 3000 rpm for 15 min (Thermo Scientific, Micro Legend 12) and then stored at -20°C until analyzed.

Determination of bone turnover marker and estradiol hormone: Using a commercial OC kit, beta βCTx and estradiol kit, OC levels (as a marker of bone formation), βCTx levels (as a marker of bone resorption) and estradiol hormone in serum were measured. All analyses were conducted adhering to the instructions of the test kit manufacturers. The results were analyzed using the ZN-320 Zenix (Briloner Landstrade, Korbach-Germany) microplate reader. The data represent the percentage change of OC, βCTx and estradiol hormone in the serum before and after the set treatment.

Statistical analysis: Data were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's Multiple-Range (DMR) test using the SPSS 18.0 Statistical Software Program (SPSS, Inc., IBM, Chicago, Illinois, USA). Differences of $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Serum osteocalcin (OC) and beta-crosslaps (βCTx): All groups of rats showed increased levels of serum osteocalcin and βCTx. There was a significant difference in the degree of change in serum levels of OC and βCTx between the OVX and N-C groups ($p<0.05$) (Table 1). The highest increase in OC and βCTx serum levels was observed in the OVX-C group. The degree of change in OC and βCTx levels in the OVX-E and OVX-SS groups was similar, yet was lower than in the OVX-C and OVX-S groups. The OVX-S group experienced a smaller increase compared to OVX-C group.

Table 1: Changes in bone turnover biochemical biomarker and estradiol on ovariectomized rats administered with soybean flour, soybean sprout flour and ethynilestradiol

Groups	Changes (%)		
treatment	Osteocalcin	Beta crosslaps (βCTx)	Estradiol
N-C	3.69±2.25^{a}	7.06±2.47^{a}	2.51±9.19^{d}
OVX-C	20.60±2.52^{d}	102.59±6.86^{c}	-52.28±6.85^{a}
OVX-E	10.96±0.69^{b}	54.77±6.40^{b}	-18.60±6.87^{c}
OVX-S	17.25±2.40^{c}	97.29±12.76^{c}	-39.35±8.79^{b}
OVX-SS	12.73±1.04^{b}	63.26±7.09^{b}	-32.15±10.05^{b}

NC: Normal control, OVX-C: Ovariectomized control, OVX-E: Ovariectomized +estradiol, OVX-S: Ovariectomized+soybean flour, OVX-SS: Ovariectomized +soybean sprout flour. Data were analyzed using ANOVA, values are Mean±SD. Values with different superscripts within a column are significantly different at $p\leq0.05$ by Duncan's Multiple-Range (DMR) test

OC and βCTx are activity markers of osteoblasts (bone formation) and osteoclasts (bone resorption) and are found in blood serum. Both types of cells play a role in bone turnover, thus high levels of these markers indicate high bone turnover activity. High bone turnover can lead to the loss of bone mass. As explained by Lelovas *et al.*[20], after the rats are ovariectomized, they experience high activity of bone resorption, leading to bone mass loss. According to Hoegh-Andersen *et al.*[21] estrogen deficiency induced by ovariectomy will lead to decrease in bone mass due to increased activity of osteoclasts. Lim and Kim[22] found that four weeks after ovariectomy, rats experienced significant deterioration in bone mass.

High levels of βCTx indicated excessive osteoclast cell activity, which was followed by high osteoblast cell activity[23,24]. The results of this study also indicated high bone turnover in the ovariectomized group compared with the control group. These results explain the minimal change in OC and βCTx levels in the OVX-E and OVX-SS groups compared to the ovariectomized groups. The increase in βCTx levels was similar in the OVX-S and OVC-C groups.

Increased serum concentrations of OC and βCTx are related to low estrogen levels secondary to ovariectomy in rats. Researchers have previously reported similar findings[22,25]. According to Wafay *et al.*[10], decreased estrogen led to increased bone turnover. Estrogen lowered differentiation of osteoclast progenitor cells, inhibited bone resorption activity and induced apoptosis of mature osteoclasts[26]. Administering estradiol or isoflavones to ovariectomized rats can inhibits the increase of both OC and βCTx[22,25], leading to a reduction in bone turnover and loss.

Beneficial effects of legume isoflavones on the activity of osteoclasts and osteoblasts in osteoporosis rat models have been previously reported. Soybean isoflavones are known to induce bone formation through several mechanisms, including stimulation of activity, proliferation and differentiation of osteoblast cells[27,28] protecting osteoblasts from apoptosis and encouraging bone formation. Isoflavones also helped suppress osteoclast cell activity and increase the activity of osteoblasts[29]. Byun and Lee's work[30] also demonstrates that isoflavones are effective in decreasing bone loss in ovariectomized rats.

The role of soybean isoflavones on balancing the activity of osteoclasts and osteoblasts (rate of bone turnover) depends on the dose and duration of consumption. Picherit *et al.*[31] suggested that isoflavones may decrease bone turnover through dose-dependent anti-osteoclastic activity. A study by Wafay *et al.*[10] showed that treating ovariectomized rats with a phyto soya extract at 30.6 mg day^{-1} for 12 weeks significantly lowered serum osteocalcin (12.93±1.13 ng dL^{-1}) compared

with a control group (19.77±2.08 ng dL^{-1}). Data obtained by Picherit *et al.*[31] showed that isoflavones could prevent the decrease in Bone Mass Density (BMD) in ovariectomized rats after 13 weeks of treatment. Likewise, Jeon *et al.*[32] discovered that BMD is significantly different in ovariectomized rats after administration of isoflavone treatments for 19 weeks.

Administering soybean sprout flour to ovariectomized rats did increase their serum OC and βCTx, but the improvement was more significant in rats receiving soybean flour. The presence of components other than isoflavones in soybean sprout flour may have contributed to the suppression of bone turnover activity. Soybean sprouts are known to have higher level of antioxidant components than soybeans, i.e., phenolic, α-tocopherol and vitamin C[16]. Ovariectomy in rats also causes oxidative stress, which decreases bone mineral density[33,34]. Norazlina *et al.*[35] reported that Reactive Oxygen Species (ROS) can accumulate in ovariectomized rats, causing stress oxidation, an increase in osteoclast cells and bone loss. Antioxidant components play an important role in the suppression of osteoclast cell activity secondary to oxidative stress in ovariectomized rats[33].

Arslan *et al.*[34] showed that ovariectomized rats supplemented with vitamin C have lower markers of oxidative stress than those in control groups. Setiawan *et al.*[36] reported that administration of soy sprouts at 2.12 g/day for 4 weeks can lower markers of oxidative stress in Sprague Dawley rats with hypercholesterolemia by decreasing levels of malonaldehyde (MDA) and increasing levels of superoxide dismutase (SOD).

Serum estradiol level: Serum estradiol levels in the OVX group of rats significantly decreased compared to those the N-C group of rats, especially because levels in the N-C group of rats increased (Table 1). The decline of serum estradiol in the OVX-C group was significantly higher than in the other OVX group; the lowest decline was seen in the OVX-E group, while the OVX-S and OVX-SS goups showed no difference.

These results offer several impressions. First, rat serum estradiol levels increased after ovariectomy with the treatment given to the OVX-E; OVX-S and OVX-SS groups. Second, the dose given to rats for 6 weeks was not sufficient to prevent estrogen depletion secondary to ovariectomy.

Estrogen hormone is produced by cells in the lutein, which is located in the ovarian theca interna[37]. Therefore, ovariectomy directly causes a decrease in estrogen production[38,26]. The results of this study are similar with those obtained by Lee *et al.*[26], showing no significant difference between serum estradiol levels in groups of ovariectomized rats given legumes with serum estradiol levels in an ovariectomized control group. In the previous study, four types of legumes had been given to four different groups of rats for 10 weeks, with legumes (cornstarch) replacing as much as 35% of the rats' feed. Treatment using estradiol showed different results. Modder *et al.*[39] demonstrated that the serum estradiol level of ovariectomized rats given estradiol at 5-20 μg kg^{-1}/day for 1 month was similar to that of non-ovariectomized rats. This is similar to data obtained by Lim *et al.*[24] showing that the provision of 17 β-estradiol at 10 μg kg^{-1}/day for 8 weeks significantly increased the serum estradiol level more than that in a control group. Treatment using isoflavones extracted from *P. lobata* administered orally at a dose of 30 mg kg^{-1}/day brought no effect, yet at a higher doses of 100 mg kg^{-1}/day, serum estradiol increased significantly more than in a control group. Lim *et al.*[24] suspected that phytoestrogen supplementation in high doses could improve the level of estradiol via activation of estrogen by sulfotransferase (SULT1E1) gene expression in ovariectomized rats. These studies appear to demonstrate that dose and duration of administration of estradiol and/or isoflavones affect the serum estradiol levels in ovariectomized rats.

CONCLUSION

The results of the study showed that soybean sprout flour consumption provided a better effect on the inhibition of bone turnover activity in ovariectomized rats than soybeans did. The potential of soybean and soybean sprouts in estradiol hormone recovery on ovariectomized rats was not different.

These results indicate that soy sprouts have a positive effect on the reduction of bone loss in ovariectomized rats. It is suggested that consumption of soybean sprout flour on a regular basis could help to reduce the loss of bone mass in postmenopausal women.

SIGNIFICANCE STATEMENT

This study discovers the possible synergistic effect of isoflavones and other antioxidants in sprouts such as vitamin C, vitamin E and polyphenol in the prevention of bone loss in ovariectomized rats. Other combinations that may be more effective for the prevention of osteoporosis in a postmenopausal rat model are isoflavones, antioxidant compounds, calcium, vitamin D and physical exercise. This study will help the researcher to uncover the critical area of postmenopausal bone loss that many researchers were not able to explore. Thus, new theories on combinations of isoflavones, antioxidant compounds and calcium as well as other combinations may be discovered.

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REFERENCES

1. NOF., 2013. Clinician's guide to prevention and treatment of osteoporosis. National Osteoporosis Foundation, Washington, DC., pp: 1-53.
2. Dhanwal, D.K., C. Cooper and E.M. Dennison, 2010. Geographic variation in osteoporotic hip fracture incidence: The growing importance of Asian influences in coming decades. J. Osteoporosis. 10.4061/2010/757102.
3. Micosch, P., 2012. Osteoporosis: Pathophysiology and Clinical Aspects. In: Principles of Osteoimmunology Molecular Mechanisms and Clinical Applications, Pietschaman, P. (Ed.)., Springer, New York, pp: 137-162.
4. Nilsson, S. and J.A. Gustafsson, 2002. Biological role of estrogen and estrogen receptors. Crit. Rev. Biochem. Mol. Biol., 37: 1-28.
5. Biver, E., F. Chopin, G. Coiffier, T.F. Brentano, B. Bouvard, P. Garnero and B. Cortet, 2012. Bone turnover markers for osteoporotic status assessment? A systematic review of their diagnosis value at baseline in osteoporosis. Joint Bone Spine, 79: 20-25.
6. King, J., C.H. Wynne, L. Assersohn and A. Jones, 2011. Hormone replacement therapy and women with premature menopause: A cancer survivorship issue. Eur. J. Cancer, 47: 1623-1632.
7. Cui, Y., S. Deming-Halverson, A. Beeghly-Fadiel, L. Lipworth and M.J. Shrubsole *et al*., 2014. Interactions of hormone replacement therapy, body weight and bilateral oophorectomy in breast cancer risk. Clin. Cancer Res., 20: 1169-1178.
8. Borrelli, F. and E. Ernst, 2010. Alternative and complementary therapies for the menopause. Maturitas, 66: 333-343.
9. Wang, Q., X. Ge, X. Tian, Y. Zhang, J. Zhang and P. Zhang, 2013. Soy isoflavone: The multipurpose phytochemical. Biomed. Rep., 1: 697-701.
10. Wafay, H.A., M. Abdel-Moniem, H.A. Megahed and H. Elmalt, 2013. The effect of soy isoflavones and nondigestive oligosccharides on bone turnover markers. Int. J. Pharm. Pharm. Sci., 5: 152-156.
11. Park, Y., H.J. Moon, D.J. Paik and D.Y. Kim, 2013. Effect of dietary legumes on bone-specific gene expression in ovariectomized rats. Nutr. Res. Pract., 7: 185-191.
12. Alekel, D.L., M.D. van Loan, K.J. Koehler, L.N. Hanson and J.W. Stewart *et al*., 2010. The soy isoflavones for reducing bone loss (SIRBL) study: A 3-y randomized controlled trial in postmenopausal women. Am. J. Clin. Nutr., 91: 218-230.
13. Oseni, O.A., A.U. Ibeto and M.O. Aruna, 2011. Effects of dehusking on the composition of phytochemicals nutrients, antinutrients, minerals and *in-vitro* multi enzyme digestibility of the seed of Brazilian Jack beans (*Canavalia braziliensis*). Int. Res. J. Biotechnol., 2: 192-197.
14. Afiukwa, C.A., O. Onwuchekwa, U.A. Ibiam, C.O. Edeogu and P.M. Aja, 2012. Characterization of cowpea cultivars for variations in seed contents of some Antinutritional Factors (ANFs). Cont. J. Food Sci. Technol., 6: 25-34.
15. Mugendi, J.B., E.N.M. Njag, E.N. Kuria, M.A. Mwasaru, J.G. Mureithi and Z. Apostolides, 2010. Effects of processing techniques on the nutritional composition and anti-nutrient content of mucuna bean (*Mucuna pruriens* L.). Afr. J. Food Sci., 4: 156-166.
16. Li, L., B. Liu and X. Zheng, 2011. Bioactive ingredients in adzuki bean sprouts. J. Med. Plants Res., 5: 5894-5898.
17. Rusydi, M.R.M. and A. Azrina, 2012. Effect of germination on total phenolic, tannin and phytic acid contents in soy bean and peanut. Int. Food Res. J., 19: 673-677.
18. Hartiningsih, D. Anggraini and D. Aji, 2012. Respon metafisis tulang femur distalis tikus ovariektomi yang mengkonsumsi kalsitriol. J. Kedokteran Hewan, 6: 92-97.
19. Reeves, P.G., F.H. Nielsen and G.C. Fahey Jr., 1993. AIN-93 purified diets for laboratory rodents: Final report of the American institute of nutrition *Ad hoc* writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr., 123: 1939-1951.
20. Lelovas, P.P., T.T. Xanthos, S.E. Thoma, G.P. Lyritis and I.A. Dontas, 2008. The laboratory rat as an animal model for osteoporosis research. Comp. Med., 58: 424-430.
21. Hoegh-Andersen, P., L.B. Tanko, T.L. Andersen, C.V. Lundberg and J.A. Mo *et al*., 2004. Ovariectomized rats as a model of postmenopausal osteoarthritis: Validation and application. Arthritis Res. Ther., Vol. 6. 10.1186/ar1152.
22. Lim, D.W. and Y.T. Kim, 2014. Anti-osteoporotic effects of *Angelica sinensis* (Oliv.) Diels extract on ovariectomized rats and its oral toxicity in rats. Nutrients, 6: 4362-4372.
23. Hertrampf, T., B. Schleipen, C. Offermanns, M. Velders, U. Laudenbach and P. Diel, 2009. Comparison of the bone protective effects of an isoflavone-rich diet with dietary and subcutaneous administrations of genistein in ovariectomized rats. Toxicol. Lett., 184: 198-203.
24. Lim, D.W., J.G. Kim and Y.T. Kim, 2013. Effects of dietary isoflavones from Puerariae radix on lipid and bone metabolism in ovariectomized rats. Nutirent, 5: 2734-2746.
25. Miao, Q., J.G. Li, S. Miao, N. Hu and J. Zhang *et al*., 2011. The bone-protective effect of genistein in the animal model of bilateral ovariectomy: Roles of phytoestrogens and PTH/PTHR1 against post-menopausal osteoporosis. Int. J. Mol. Sci., 13: 56-70.

26. Lerner, U.H., 2006. Bone remodeling in post-menopausal osteoporosis. J. Dent. Res., 85: 584-595.
27. Lee, Y.S., X. Chen and J.J.B. Anderson, 2001. Physiological concentrations of genistein stimulate the proliferation and protect against free radical-induced oxidative damage of MC3T3-E1 osteoblast-like cells. Nutr. Res., 21: 1287-1298.
28. Chen, X.W., S.C. Garner and J.J.B. Anderson, 2002. Isoflavones regulate interleukin-6 and osteoprotegerin synthesis during osteoblast cell differentiation via an estrogen-receptor-dependent pathway. Biochem. Biophys. Res. Commun., 295: 417-422.
29. Chiang, S.S. and T.M. Pan, 2013. Beneficial effects of phytoestrogens and their metabolites produced by intestinal microflora on bone health. Applied Microbiol. Biotechnol., 97: 1489-1500.
30. Byun, J.S. and S.S. Lee, 2010. Effect of soybeans and sword beans on bone metabolism in a rat model of osteoporosis. Ann. Nutr. Metab., 56: 106-112.
31. Picherit, C., C. Bennetau-Pelissero, B. Chanteranne, P. Lebecque, M.J. Davicco, J.P. Barlet and V. Coxam, 2001. Soybean isoflavones dose-dependently reduce bone turnover but do not reverse established osteopenia in adult ovariectomized rats. J. Nutr., 131: 723-728.
32. Jeon, B.J., J. Ahn and H.S. Kwak, 2009. Effect of Isoflavone-enriched milk on bone mass in ovariectomized rats. J. Med. Food, 12: 1260-1267.
33. Muthusami, S., I. Ramachandra, B. Muthusamy, G. Vasudevan and V. Prabhu *et al*., 2005. Ovariectomy induces oxidative stress and impairs bone antioxidant system in adult rats. Clin. Chim. Acta, 360: 81-86.
34. Arslan, A., S. Orkun, G. Aydin, I. Keles, A. Tosun, M. Arslan and O. Caglayan, 2011. Effects of ovariectomy and ascorbic acid supplement on oxidative stress parameters and bone mineral density in rats. Libyan J. Med., 10.3402/ljm.v6i0.5965.
35. Norazlina, M., H. Hermizi, O. Faizah, A.N. Shuid, M. Norliza and S. Ima-Nirwana, 2010. Vitamin E reversed nicotine-induced toxic effects on bone biochemical markers in male rats. Arch. Med. Sci., 6: 505-512.
36. Setiawan, D.I., K. Tjahyono and D.N. Afifah, 2016. Pemberian kecambah kacang kedelai terhadap kadar malondialdehid (MDA) dan superoxide dismutase (SOD) tikus Sprague Dawley hiperkolesterolemia. J. Gizi Klinik Indonesia, 13: 20-26.
37. Price, S.A. and L.M. Wilson, 1994. Patofisiologi: Konsep Klinis Proses-Proses Penyakit. 4th Edn., Chapter 64, Buku Kedokteran EGC., Jakarta, pp: 1125-1157.
38. Hong, G.E., P.K. Mandal, C.W. Pyun, K. Choi and S.K. Kim *et al*., 2009. Isoflavone aglycone from fermented soy pulp prevents osteoporosis in ovariectomized rats. Asian J. Anim. Vet. Adv., 4: 288-296.
39. Modder, U.I., B.L. Riggs, T.C. Spelsberg, D.G. Fraser, E.J. Atkinson, R. Arnold and S. Khosla, 2004. Dose-response of estrogen on bone versus the uterus in ovariectomized mice. Eur. J. Endocrinol., 151: 503-510.