Anti-Osteoporotic Activity of Soy Total Extract and Genistein Compound in Ovariectomized Rats

Dalia A. Hafez

Nutrition and Food Science Department, Faculty of Design and Home Economics, Taif University, Kingdom of Saudi Arabia
Home Economics Department, Faculty of Education, Suez Canal University, Egypt
daliaij_2000@yahoo.com

Abstract: This study was undertaken to investigate the anti-osteoporotic activity of soy total extract and genistein isoflavone in ovariectomized (OVX) rats. Bilateral ovariectomy was performed in rats under aseptic condition and ether anesthesia. Rats were randomly distributed into 6 equal groups of 7 animals each. Group (I) was sham operated and the other 5 groups were OVX. Group (II) was left OVX control, while groups (III), (IV), (V) and (VI) were given orally soy extract in 250 and 500mg/kg^{-1} and genistein in 25 and 50 mg/kg^{-1}, respectively for 8 weeks. At end of the experiment, blood was collected for estimating serum calcium (Ca), phosphorous (P), bone - specific alkaline phosphatase (b-ALP) and osteocalcin (OC). Urine samples were collected for determining Ca and P concentrations. Rats were euthanized and the uteri were removed and weighed. Both femur bones were taken for bone analysis. The results showed that ovariectomy caused increases in serum levels of Ca, P, b-ALP and OC and decreases in uterine and femur weights. Administration of soy extract and genistein to OVX rats normalized the elevated serum levels of Ca, P, b-ALP and OC and restored uterine and femur weights. Ovariectomy also increased urinary Ca and P levels and decreased femur volume, mineral density and calcium content in bone ash. Treatment with soy extract and genistein normalized urinary Ca and P levels and increased femur bone volume, density and calcium content. In conclusion, total soy extract has an anti-osteoporotic activity in ovariectomized rats. This study recommends that intake of soybean in foods may be beneficial as an alternative therapy for women who suffer from postmenopausal osteoporosis.
[Dalia A. Hafez **Anti-Osteoporotic Activity of Soy Total Extract and Genistein Compound in Ovariectomized Rats.** *J Am Sci* 2012;8(8):698-703]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 106

Keywords: Soybean; Genistein; Osteoporosis; Calcium, Phosphorous; Osteocalcin; Ovariectomized rats.

1. Introduction

Osteoporosis is a bone disease characterized by bone mass reduction, deterioration of bone structure and decreased bone mineral density which leads to an increased risk of bone fractures. The bone matrix is composed of organic and inorganic components. The organic components include collagen and glycoprotein and the inorganic include minerals mainly calcium and phosphorus. Both organic and inorganic components provide strictness and strength to the bones **(Annemieke *et al.,* 1997)**. Osteoporosis represents a major public health problem among general populations particularly in postmenopausal women due to loss in estrogen level (**Vassilopoulou-Sellin, 2003)**.

Phytoestrogens are plant-derived compounds which include isoflavones and lignans. These compounds are found in many foods and are structurally and functionally similar to estrogen. Phytoestrogens have been reported to produce both weak estrogenic effect by acting on estrogen receptors and antiestrogenic action; hence they are termed natural selective estrogen receptor modulators (**Basly and Lavier, 2005)**.

Soybeans contain two main types of isoflavones namely genistein and daidzein. Isoflavones are part of a large group of plant chemicals called flavonoids which are found in many fruits, vegetables and legumes. Soybeans are the most concentrated source of isoflavones in the human diet. Isoflavones have similar structure to estrogen and have the ability to exert both estrogenic and antiestrogenic effects. They may block the effects of estrogen in some tissues such as endometrium and the breast, but they act similar to estrogen in providing possible protection against bone loss and heart diseases **(Gris Martinez, 2006)**. The consumption of soy foods has been linked to human health benefits such as lowering serum cholesterol so inhibit the development of atherosclerosis **(Nagata and Yamada, 2009)**; producing antioxidant properties **(Salih *et al.,* 2009)**; protecting against breast and prostate cancer **(Nagata, 2010)**; relieving the discomforts of menopause and may be beneficial in treating menopausal symptoms and reducing the risk of osteoporosis **(Byun and Lee, 2010)**.

Genistein isoflavone has been demonstrated to induce weak estrogenic, antioxidant, hypotensive and anticancer actions. Therefore it has been associated with beneficial effects in the treatment of osteoporosis, hypertension and some types of cancer **(Rivas *et al.,* 2002 and Tiulea *et al.*, 2011)**.

The aim of the present study was to examine the anti-osteoporotic activity of total soy extract and genistein isoflavone in ovariectomized rats.

2. Material and Methods

2.1. Soybeans:

Dry soybean (Family *Fabaceae* or *Leguminosae*) seeds were purchased from Agricultural Seeds, Herbs and Medicinal Plant Company, Cairo, Egypt. The seeds were authenticated by a taxonomist at the Botany Department, Faculty of Agriculture, Cairo University. The dry seeds were grinded in an electric grinder into a fine powder.

2.2. Genistein:

Genistein (4, 5, 7-trihydroxyflavone) is found in a high content in soybeans and soy products. It was supplied as a pure powder from Extrasynthèse Company (France). Genistein was prepared as a suspension with hydroxypropyl cyclodextrin (Cyclolab Co., Hungaria) in a ratio of 1:2 because of its poor water solubility (**Cannava *et al.*, 2010)**.

2.3. Rats:

Forty two adult female rats of Sprague Dawley strain weighing 200-210 g body weight and 14-16 weeks old were used in this study. The rats were obtained from the Laboratory Animal Colony, Helwan, Egypt. The animals were housed individually under hygienic conditions in metabolic cages. The rats were kept at a room temperature of 25 ± 2 °C with relative humidity of 50–60% and on 12 hrs light/12 hrs dark cycles in the Animal House at the Agricultural Research Center, Giza, Egypt.

2.4. Preparation of basal diet:

Basal diet was prepared according to **Reeves *et al.* (1993)**. It consists of 20 % protein, 10 % sucrose, 4.7% corn oil, 2% choline chloride, 1% vitamins mixture, 3.5 % salt mixture and 5% fibers. The remainder was corn starch up to 100 %.

2.5. Preparation of soy total extract:

The total extract of soybean seeds was prepared according to the method described by **Shalaby and Hamowieh (2010)**. Five hundred grams of powdered soy seeds were soaked in one liter of 90% ethyl alcohol and kept in a refrigerator with daily shaking for 5 days. The extract was filtered through double layers of gauze and ethanol was evaporated on a rotatory evaporator (Model No.570, West Germany) at 50°C. After evaporation, the yielded amount was 126.52 grams of crude semisolid extract. Twenty grams of the crude extract were suspended in 2 ml of Tween 80 (suspending agent) and distilled water was added up to 100 ml to obtain 20% soy total liquid extract.

2.6. Ovariectomy procedure:

Ovariectomy was performed in experimental groups of rats via bilateral incisions at both right and left flanks under ether anesthesia. The periovarian fatty tissue was identified and exteriorized. For prevention of subsequent hemorrhage, the arterial blood vessels were compressed by an artery forceps. The ovaries were removed out by cutting above the clamped area and uterine horns and blood vesicles were legated. Muscles and skin were stitched separately (**Shalaby, 1977)**. Similarly, sham operation was performed in rats where the ovaries were exposed but not removed. Povidone-iodine antiseptic solution was applied locally on the skin as disinfectant.

2.7. Experiment and grouping of rats:

After complete surgical recovery, the operated rats (n = 42) were randomly distributed into 6 equal groups of 7 rats each. Group (I) was sham - operated (SHAM) and the other five groups were ovariectomized (OVX) and left after surgical operation for 3 weeks to ensure almost complete clearance of their bodies from estrogen hormone residues. At beginning and end of the experiment period (8 weeks), the rats were weighed and the changes in body weight gain were calculated. Group (II) was left OVX control non treated, while groups (III) and (IV) were orally given soy total extract in 250 and 500 $mg.kg^{-1}$/ day for 8 weeks, respectively. Groups (V) and (VI) were orally given genistein compound as a suspension in 25 and 50 $mg.kg^{-1}$/ day for the same period, respectively. The selected doses of genistein were calculated for the rat from human equivalent dose according to **Paget and Barnes (1964)**. At end of the experiment, blood and urine samples were collected for biochemical analyses. Both uterine horns of each rat were removed and weighed. Both femur bones of each rat were removed and taken for bone analyses. The experiment was carried out according to rules and guidelines for animal experimentation which approved by the Institutional Animal Care and Use Committee, National Research Centre, Dokki, Egypt.

2.8. Biochemical analyses:

Blood samples were collected from portal hepatic vein into clean dry centrifuge tubes and centrifuged at 3000 rpm for 10 minutes to separate the serum which was stored in a refrigerator till biochemical analyses. Urine samples were collected then acidified with 12 Mol. HCL and stored in a refrigerator till analyses. Concentrations of calcium **(Gindler and King, 1972)** and phosphorus **(Goodwin, 1970)** in both serum and urine were determined spectrophotometrically using specific diagnostic reagent kits (BioMérieux, France). Serum bone-specific alkaline phosphate **(Roy, 1970)** was estimated by colorimetric assay using specific enzyme kits (Sigma Co., St. Louis, USA). Serum osteocalcin concentration was measured by an enzyme-linked immunosorbent assay (Rat Mid™, Osteocalcin ELISA kit, IDS Inc., Fountain Hills,

Arizona, USA) according to the manufacturer's instructions.

2.9. Determination of femur bone weight, length, volume and density:

The soft tissues around the right femur bone were removed and the femur was weighed. Femur length was measured with vernier caliper and femur volume and density were calculated using Archimedes' principle according to **Doyle and Cashman (2003)**. In brief, the femur was cut out at the mid diaphyses and bone marrow was washed out. Each bone was placed in an unstoppered vial filled with deionized water, and the vial was placed for 90 minutes in a vacuum desiccator. The desiccator was agitated periodically to ensure that the trapped air completely diffused out. The bone was removed from the vial, dried by blotted paper, weighed, and placed again in the vial containing deionized water. The bone was reweighed in a suspended vessel and should be not completely immersed in water before equilibrated at room temperature. Femur bone density (bone weight/bone volume) was then calculated.

2.10. Determination of femur bone mineral content:

The left femur bone was dried overnight at 100^{o}C. The femur was then incinerated for 12 hrs at 1000^{o}C in Muffle apparatus to obtain the ash. Then, the ash was weighed, solubilized with 6 N HCL **(Yang *et al.*, 2008)** and quantitatively transferred into volumetric flask then completed to 100 ml with 6 N HCL. The solutions were used for analysis of calcium content in bone ash using atomic absorption spectrophotometer (model 2380, Perkin-Elmer, Wellesley, MA, USA). The phosphorus content in bone ash was determined using spectrophotometer (UV 2000, Hitachi Ltd., Tokyo, Japan).

2.11. Statistical analysis:

Data are expressed as mean ± SE. Differences between all experimental groups were tested for significance using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences were considered significant at P <0.05 according to **Snedecor and Cochran (1986)** using computerized SPSS program.

3. Results

The overall body weight analysis at end of the experiment (8 weeks) revealed that the ovariectomized (OVX) control rats had a significant (P <0.05) increase in body weight gain. The body weight gain was 28.86% for OVX rats versus to18.34% for sham (SHAM) control group. Ovariectomy caused a significant (P <0.05) decrease in uterine weight when compared with SHAM control rats. The mean uterine weight of rats was 0.19±0.01 g in OVX control group and 0.39±0.04 g in SHAM group. Oral administration of the small and large doses (250 and 500 mg/kg^{-1}) of soy total extract or genistein (25 and 50 mg/kg^{-1}) to OVX rats reversed the changes (P <0.05) in both body weight gain and uterine weight when compared with the OVX control group as shown in Table (1).

The bilateral ovariectomy in rats resulted in significant (P < 0.05) increases in serum levels of calcium, phosphorous, bone-specific alkaline phosphatase (b-ALP) and osteocalcin (OC) when compared with the SHAM group. The treatment of OVX rats with the small and large doses (250 and 500 mg/kg^{-1}) of soy total extract and genistein (25 and 50 mg/kg^{-1}) significantly (P < 0.05) normalized the elevated serum levels of Ca, P, b-ALP and OC when compared with the OVX control group (Table 2). The analysis of urine samples of OVX control rats revealed a significant (P < 0.05) increase in calcium and phosphorous levels when compared with SHAM control rats. Oral administration of soy total extract (250 and500 mg/kg^{-1}) and genistein (25 and 50 mg/kg^{-1}) to OVX rats significantly (P < 0.05) reduced the elevated levels of calcium and phosphorous in the urine when compared to the OVX-control group (Table 3).

As shown in Table (4) the ovariectomized control rats had a significant (P < 0.05) decrease in femur weight, volume and mineral density when compared with the SHAM control group. The treatment of OVX rats with soy total extract (250 and500 mg/kg^{-1}) and genistein (25 and 50 mg/kg^{-1}) significantly (P < 0.05) restored the ovariectomy-induced changes in femur weight, volume and mineral density when compared with OVX control rats. There were no changes in the length of femur bone in any of the treated groups. Data in Table (5) showed that the ovariectomy in rats caused a significant (P < 0.05) decrease in ash weight of femur bone and calcium content in femur ash when compared with SHAM control rats. There was no significant change in phosphorous content in femur ash of any of the treated groups. The decreases in femur ash weight and calcium content in femur ash were significantly (P < 0.05) normalized in OVX rats given orally soy extract (250 and500 mg/kg^{-1}) and genistein (25 and 50 mg/kg^{-1}).

4. Discussion

The present study was aimed to investigate the anti-osteoporotic activity of soy total extract and genistein isoflavone, each at two dosage levels, in ovariectomized rats.

It is well known that estrogen deficiency is one of the important risk factors in the pathogenesis of osteoporosis. It is also evident that the bilateral ovariectomy results in dramatic decreases in uterine weight, bone mineral content, density and biomechanical strength due to estrogen loss

(Vassilopoulou-Sellin, 2003 and Srikanta *et al.*, 2011). It was previously reported that human foods which contain phytoestrogens can be useful in the prevention and treatment of osteoporosis **(Mori et *al.*, 2004 and Coxam, 2005)**. Soybean isoflavones have similar chemical structure to estrogen and have received attention as alternatives to hormone replacement therapy for the prevention of postmenopausal osteoporosis. Phytoestrogens were found to produce a protective effect against osteoporosis due to their ability to exert an estrogen like action on bone cells in postmenopausal women via suppressing osteoclastic bone resorption and promoting osteoblastic bone formation **(Fujioka *et al.*, 2004 and Morin, 2004)**.

Table 1. Effects of soy extract (SEx) and genistein (GEN) on body and uterus weights of ovariectomized (OVX) rats.

Groups	Body weight (g) at Week 0	Body weight (g) at Week 8	Weight gain (%)	Uterine weight (g)
Group I SHAM - control	200.00±8.85[a]	226.85±5.32[c]	18.34	0.39±0.04[a]
Group II OVX - control	210.65±6.17[a]	271.46±6.17[a]	28.86	0.19±0.01[c]
Group III SEx (250 mg/kg^{-1})	208.82±5.62[a]	242.71±4.25[b]	16.22	0.25±0.03[b]
Group IV SEx (500 mg/kg^{-1})	206.54±7.25[a]	240.52±2.33[b]	16.45	0.26±0.01[b]
Group V GEN (25 mg/kg^{-1})	208.38±8.65[a]	244.75±3.63[b]	17.45	0.24±0.03[b]
Group VI GEN (50 mg/kg^{-1})	209.59±9.12[a]	247.42±2.53[b]	18.06	0.23±0.04[b]

Means ± SE with different superscripts in the same column are significant at $P < 0.05$ using one way ANOVA test. n=7rats

Table 2. Effects of soy extract (SEx) and genistein (GEN) on levels of serum calcium (Ca), phosphorous (P), bone specific alkaline phosphatase (b-ALP) and osteocalcin (OC) in ovariectomized (OVX) rats.

Groups	Concentrations (Mean ± SE) of Ca (mg/dL)	P (mg/dL)	b-ALP (IU/ml)	OC (µg/L)
Group I SHAM-control	11.09±0.8[b]	3.65±0.2[b]	158.6±9.3[b]	0.9±0.04[c]
Group II OVX-control	13.41±0.7[a]	6.16±0.1[a]	168.6±4.2[a]	1.8±0.02[a]
Group III SE (250 mg/kg^{-1})	10.15±0.4[b]	3.37±0.2[b]	142.5±5.1[c]	1.3±0.2[b]
Group IV SE (500 mg/kg^{-1})	10.25±0.6[b]	3.25±0.1[b]	146.6±6.3[c]	1.2±0.01[c]
Group V GEN (25 mg/kg^{-1})	10.64±0.8[b]	3.55±0.2[b]	150.6±7.3[c]	1.4±0.02[b]
Group VI GEN (50 mg/kg^{-1})	10.77±0.4[b]	3.65±0.1[b]	155.6±8.6[b]	1.5±0.03[b]

Means ± SE with different superscripts in the same column are significant at $P < 0.05$ using one way ANOVA test; n=7 rats.

Table 3. Effects of soy extract (SEx) and genistein (GEN) on urine levels of calcium (Ca) and phosphorus (P) in ovariectomized (OVX) rats.

Groups	Concentrations (Mean ± SE) of Ca (mg/dL)	P (mg/dL)
Group I SHAM- control	4.87±0.8[d]	9.85±0.3[b]
Group II OVX- control	8.25±0.7[a]	11.16±0.7[a]
Group III SEx (250 mg/kg^{-1})	5.25±0.4[c]	8.67±0.2[b]
Group IV SEx (500 mg/kg^{-1})	5.25±0.4[c]	8.55±0.3[b]
Group V GEN (25 mg/kg^{-1})	6.33±0.8[b]	8.35±0.5[b]
Group VI GEN (50 mg/kg^{-1})	6.44±0.4[b]	8.25±0.3[b]

Means ± SE with different superscripts in the same column are significant at $P < 0.05$ using one way ANOVA test. n=7 rats.

Table 4. Effects of soy extract (SEx) genistein (GEN) on femur weight (Wt), length (L), volume (V) and density (D) in ovariectomized (OVX) rats.

Groups	Parameters (Mean ± SE) Femur Wt. (g)	Femur L (mm)	Femur V (cm^3)	Femur D (g/cm^3)
Group I SHAM- control	0.75±0.02[b]	40.02±2.75	0.74±0.01[a]	0.84±0.04[a]
Group II OVX- control	0.66±0.17[c]	40.09±8.71	0.44±0.02[c]	0.64±0.02[c]
Group III SEx (250 mg/kg^{-1})	0.80±0.02[a]	39.80±9.25	0.52±0.01[b]	0.75±0.07[b]
Group IV SEx (500 mg/kg^{-1})	0.81±0.03[a]	39.85±8.55	0.55±0.03[b]	0.77±0.01[b]
Group V GEN (25 mg/kg^{-1})	0.74±0.05[b]	39.90±4.05	0.65±0.02[a]	0.80±0.03[a]
Group VI GEN (50 mg/kg^{-1})	0.77±0.03[b]	39.79±5.15	0.68±0.04[a]	0.83±0.02[a]

Means ± SE with different superscripts in the same column are significant at $P < 0.05$ using one way ANOVA test.
n=7 rats

Table 5. Effects of soy extract (SEx) and genistein (GEN) on concentrations of calcium and phosphorous in femur bone of ovariectomized (OVX) rats.

Groups	Bone mineral content (Mean ±SE) Ash weight (g)	calcium (mg/g ash)	phosphorous (mg/g ash)
Group I SHAM-control	0.69±0.03[a]	11.8±0.12[a]	6.50±0.54
Group II OVX- control	0.58±0.05[d]	8.6±0.22[d]	6.56±0.46
Group III SEx (250 mg/kg^{-1})	0.62±0.03[c]	9.9±0.25[c]	6.57±0.56
Group IV SEx (500 mg/kg^{-1})	0.60±0.03[c]	10.2±0.12[c]	6.55±0.41
Group V GEN (25 mg/kg^{-1})	0.64±0.05[b]	10.6±0.12[b]	6.52±0.50
Group VI GEN (50 mg/kg^{-1})	0.65±0.02[b]	11.6±0.12[b]	6.54±0.53

Means ± SE with different letters superscripts in the same column are significant at $P < 0.05$ using one way ANOVA test . n=7 rats

In the present study, daily oral administration of soy total extract (250 and500 mg/kg^{-1}) for 8 weeks significantly inhibited the increase of body weight gain induced by ovariectomy and maintained the body weight changes near the normal weight of SHAM operated rats. Soy total extract (250 and 500 mg/kg^{-1}) restored the ovariectomy-induced decrease in the uterine weight. It also normalized the biochemical changes in serum levels of calcium, phosphorus, bone – specific alkaline phosphatase and osteocalcin and in urine concentrations of calcium and phosphorus. Soy total extract also restored the ovariectomy-induced changes in femur weight, volume and density and normalized calcium content in femur bone ash. Similarly, genistein isoflavone (25 and 50 mg/kg^{-1}) has restored the ovariectomy-induced abnormalities to normal levels. These biochemical results suggest that soy total extract and genistein induce similar effects to estrogen on the bone and body weight.

Concerning the effect on weight of the uterus, it is well known that estrogen increases vascularity, growth and weight of the uterus in immature rats and mice **(Shalaby, 1977)**. Therefore, the ovariectomized rats had a decreased weight of the uterus due estrogen deficiency. However, the reported increase of the uterine weight of ovariectomized rats given soy total extract, in this study, could be explained by the anti-estrogenic effect of phytoestrogens which present in total soy extract on the endometrium via blocking estrogen receptors Concerning this explanation, **Gris Martinez (2006)** suggested that phytoestrogens may block estrogen receptors which present in some tissues as endometrium and breast and so induce an anti-estrogenic effect on the uterus and decrease the uterine weight.

In the current study, the increased body weight gain and elevated serum levels of bone- specific alkaline phosphatase (b-ALP) and osteocalcin (OC) support the observations of the previous investigations related to increased body weight and elevated serum levels of b-ALP and OC due to ovarian hormone deficiency **(Ke *et al.*, 1997, Tamir *et al.*, 2001 and Coxam, 2005)**. Oral administration of soy total extract and genistein significantly normalized the ovariectomy-induced increases in body weight and elevated serum levels of b-ALP and OC.

Calcium and phosphorus are widely accepted phenotype markers for bone formation **(Evans *et al.*, 1990)**. In the present study, the treatment with soy total extract and genistein restored the decreased serum calcium and phosphorus concentrations induced by ovariectomy to normal levels. The results also showed that bilateral ovariectomy developed bone changes similar to those seen in the estrogen-deficient osteoporotic women, most markedly is the decrease in bone density **(Gao *et al.*, 2011)**. Oral administration of soy total extract or genistein significantly improved the femur bone density and so prevented bone loss due to estrogen deficiency.

The urine analysis showed that the bilateral ovariectomy significantly increased the urinary calcium and phosphorous concentrations. Soy total extract and genistein normalized the urinary calcium and phosphorous levels in the treated rats. These findings were similar to those reported by **Coxam (2005)** and **Canalis *et al.* (2007)**.

The mechanism(s) of anti-osteoporotic activity of soy total extract could be explained by presence of isoflavones (genistein and daidzein) which are functionally and structurally similar to estrogen and act as estrogen via acting on estrogen receptors **(Basly and Lavier, 2005 and Byun and Lee, 2010)**.

In conclusion, the current results showed the beneficial effects of soy total extract and genistein when given orally to rats with ovariectomy-induced osteoporosis. This study recommends that intake of soybeans in foods could be considered as natural alternative to estrogen hormone replacement therapy for the prevention and treatment of osteoporosis due to estrogen deficiency in postmenopausal women.

Corresponding author
Dalia A. Hafez
Nutrition and Food Science Department, Faculty of Design and Home Economics, Taif University
daliaij_2000@yahoo.com

References

Annemieke, M.B.; Melanie, A.M.; Engels, G.J.; Boerma, E.P. and Sabine, M.P. (1997): Changes in bone mineral density, body composition and lipid metabolism during growth hormone (GH) treatment in children with GH deficiency. J. Clin. Endocrinol. Metabol., 82: 2423-2428.

Basly, J.P. and Lavier, M.C. (2005): Dietary phytoestrogens: potential selective estrogen enzyme modulators. Planta Medica, 71(4): 287-294.

Byun J.S. and Lee, S.S. (2010): Effect of soybeans and sword beans on bone metabolism in a rat model of osteoporosis. Ann. Nutri. Metab., 56(2): 106-112.

Canalis, E.; Giustina, A. and Bilezikian, J.P. (2007): Mechanisms of anabolic therapies for osteoporosis. N. Engl. J. Med., 357(9): 905-916.

Cannava, C.; Crupi, V.; Ficarra, P.; Guardo, M.; Majolino, D.; Mazzaglia, A. and Stancanelli, R. (2010): Physical and chemical characterization of cyclodextrin/genistein complex, J. Pharm. Biomed. Anal., 51(5):1064-1068.

Coxam, V. (2005): New advances in osteoporosis nutritional prevention. J. Med. Sci., 21(3): 297-301.

Doyle, L. and K.D. Cashman, (2003): The effect of nutrient profiles of the dietary approaches to stop

hypertension diets on blood pressure and bone metabolism and composition in normotensive and hypertensive rats. Br. J. Nutri., 89: 713- 724.

Evans, D.B.; Bunning, R.A. and Russell, R.G. (1990): The effects of recombinant human interleukin-1β on cellular proliferation and the production of prostaglandin E2, plasminogen activator, osteocalcin and alkaline phosphatase by osteoblast-like cells derived from human bone. Biochem. Biophys. Res. Commun., 166: 208-216.

Fujioka, M.; Uehara, M.; Wu, J.; Adlercreutz, H.; Suzuki, K.; Kanazawa, K.; Takeda K.; Yamada, K.; Ishimi, Y. (2004): Equol, a metabolite of daidzein, inhibits bone loss in ovariectomized mice. J. Nutri.; 134(10): 2623-2627.

Gao, S.G.; Li, K.H.; Xu, M.; Jiang, W.; Shen, H.; Luo, W.; Tian, J. and Lei, G.H. (2011): Bone turnover in passive smoking female rat: relationships to change in bone mineral density. Musculoskelet., 12:131-139.

Gindler, E. M. and King, J. D. (1972): Rapid colorimetric determination of calcium in biologic fluid with Methyl thymol blue. Am. J. Clin. Pathol.,58: 376-382.

Goodwin, J.F. (1970): Determination of serum phosphorus Clin. Chem.; 16(9): 776-780.

Gris Martinez, J.M. (2006): Isoflavones in menopause women. Med. Clin. (Barc), 127(9): 352-356.

Ke, H.Z.; Chen, H.K.; Simmons, H.A.; Crawford. D.T.; Chidsey-Frink. K.L.; Jee, W.S. and Thompson, D.D. (1997): Comparative effects of droloxifene, tamoxifen and estrogen on bone, serum cholesterol, and uterine histology in the ovariectomized rat model. Bone, 20: 31-39.

Paget, G.E. and Barnes, I.M. (1964): Interspecies dosage conversion scheme in evaluation of results in different species. In: Laurence, D.R., Bacharach, A.L. (Eds.), Evaluation of Drug Activities: Pharmacometrics, vol. I. Academic Press, London, UK, Page 160-165.

Mori, M.; Aizawa, T.; Tokoro M.; Miki, T. and Yamori, Y. (2004): Soy isoflavone tablets reduce osteoporosis risk and obesity in middle –aged Japanese women. Clin. Exper. Pharmacol. Physiol.; 31(2): 39-41.

Morin S. (2004): Isoflavones and bone health. Menopause; 11(3): 239-241.

Nagata, C. (2010): Factors to consider in the association between soy isoflavone intake and breast cancer risk. J. Epidemiol., 20(2): 83-89.

Nagata, J. and Yamada, K. (2009): Effects of simultaneous intake of soybean protein and diacylglycerol on lipid profiles and body fat accumulation in rats. Biosci. Biotechnol. Biochem., 73(6): 1328-1332.

Rivas, M.; Garay, R.P.; Escanero, J.F.; Cia, P. Jr.; Cia, P. and Alda, J.O. (2002): Soy milk lowers blood pressure in men and women with mild to moderate essential hypertension. J. Nutri., 132(7): 1900-1902.

Reeves, P.G.; Nielson, F.H. and Fahmy, G.C. (1993): Reports of the American Institute of Nutrition, adhoc willing committee on reformulation of the AIN 93, Rodent diet. J. Nutri., 123: 1939-1951.

Roy, S.E. (1970): Colorimetric determination of serum alkaline phosphatase. Clin Chem.; 16:431-432.

Salih, M.S.; Nallasamy,P, P. P.; Muniyandi, P.; Perivasami,V. and Carani, V.A. (2009): Genistein improves liver function and attenuates non-alcoholic fatty liver disease in a rat model of insulin resistance. J. Diabetes, 4: 278-287.

Shalaby, M.A. (1977): Pharmacological studies on sex hormone- like effects of some Egyptian leguminous plants with their action on the uterus. M.V.Sc. Thesis submitted to Pharmacology Department, Faculty of Veterinary Medicine, Cairo University. Egypt.

Shalaby, M.A. and Hamowieh, A.R. (2010): Safety and efficacy of *Zingiber officinale* roots on fertility of male diabetic rats. Food Chem. Toxicol.; 48:2920-2924.

Snedecor, G.W. and Cochran, W.G. (1986): Statistical Methods, 7th Edition, Iowa State University Press, Ames, USA, Page 90.

Srikanta, P.; Nagarajappa, S.H.; Viswanatha, G.L.; Handral, M.; Subbanna, R.; Srinath, R.; Hiremath, G. (2011): Anti-osteoporotic activity of methanolic extract of an Indian herbal formula (NR/CAL/06) in ovariectomized rats. J. Chinese Integr. Med.; 9(10): 1125-1132.

Tamir, S.; Eizenberg, M.; Somjen, D.; Izrael, S. and Vaya J. (2001): Estrogen-like activity of glabrene and other constituents isolated from licorice root. J. Steroid Biochem. Mol. Biol., 78(3): 291-298.

Tiulea, C.; Peev, C.; Brezovan, D.; Dehelean, C. and Motoc, A. (2011): A comparison regarding antiproliferative action between soy total extract and genistein. Rom. J. Morphologic. Embryol., 52(3): 1065-1069.

Vassilopoulou-Sellin R. (2003): Breast cancer and hormonal replacement therapy. Ann. N.Y. Acad. Sci., 997:341-350.

Yang, L.; Wu, J.; Ho, L.; Yang, G. S.; Hung, Y. and Lin, W. (2008): Effect of poly-(α glutamic acid on calcium absorption in rats. Biosci. Biotechonol. Biochem., 72: 3084-3090.

7/20/2012