ORIGINAL ARTICLE

Evaluation of antiosteoporosis activity of ethanolic extract of *Punica granatum* Linn. seeds in ovariectomized-induced osteoporosis rats

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Abstract

Aim: Osteoporosis is a common disease worldwide and characterized by low bone mass and the progressive destruction of bone microstructure, resulting in an increased bone fragility and risk of fracture. The objective of this study was to evaluate the anti-osteoporotic activity of ethanolic extract of *Punica granatum* seeds (EPGS) in ovariectomized (OVX) rat model of osteoporosis using three different doses of 100, 300, and 500 mg/kg body weight per day. **Materials and Methods:** Healthy female albino rats were divided into six groups (n = 6). The first group was sham-operated normal and the remaining groups were OVX. Group 2 was fed with equivolume of saline and served as OVX control. Groups 3–6 were orally treated with estrogen (2 mg/kg) and EPGS (100, 300, and 500 mg/kg), for 90 days, respectively. The biomechanical, biochemical, and histopathological parameters were evaluated. **Results and Discussion:** Compared to the OVX control group, treatment groups showed increased femoral length, weight, volume, density ($P < 0.001$), and fourth lumbar hardness ($P < 0.001$). In addition, statistically significant changes were observed in biochemical parameters. There was a significant ($P < 0.001$) increase in ash, ash weight, and calcium level which was observed in the femoral bone of OVX rats. Histopathological evaluation of the femur section of EPGS treated groups showed significant restorative progress with increased ossification and mineralization. **Conclusions:** This study demonstrated that the EPGS had a significant therapeutic activity over osteoporosis associated with estrogen deficiency.

Key words: Bone mineral density, osteoporosis, ovariectomy, *Punica granatum*

INTRODUCTION

Osteoporosis is a chronic, progressive disease of the skeleton characterized by bone fragility due to a reduction in bone mass and possibly to alteration in bone architecture that leads to a propensity to fracture with minimum trauma.[1] The loss of bone has been attributed to an imbalance between bone formation and bone resorption. The type of osteoporosis is associated with ovarian hormone deficiency following menopause is currently the most common cause of age-associated bone loss.[2] Many synthetic agents, including estrogens in hormone replacement therapy, and selective estrogen receptor modulators such as raloxifene, droloxifen, bisphosphonates, and calcitonin, have been developed to treat osteoporosis, but each of the disassociated with adverse events such as hypercalcemia, hypercalciuria, increased risk of endometrial and breast cancer, breast tenderness, menstruation, thromboembolic events, vaginal bleeding, and hot flashes.[3] Hence, it would be most helpful to explore

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naturally occurring substances, especially of plant origin, that could prevent bone loss and are free from any adverse effects.

The incidence of osteoporosis is about 2-4 times higher in women than in men in most of the countries. A sharp decrease in ovarian estrogen production is the predominant cause of rapid, hormone-related bone loss during the first decade after menopause.[4] Menopause, aging and hereditary factors, inadequate calcium intake and absorption, lack of exercise, prolonged steroid administration, excessive alcohol intake, and cigarette smoking are the major risk factors that predispose osteoporosis.[5] The ovariectomized (OVX) rat is a convenient and reliable model for studying the efficacy of pharmaceutical agents in postmenopausal osteoporosis.[6] The pharmacological agents used to manage osteoporosis act by decreasing the rate of bone resorption, thereby slowing the rate of bone loss, or by promoting bone formation. To overcome the wide range of side effects produced by these synthetic drugs, there is an increasing demand for "green medicines" which are thought to be healthier and safer for the treatment of osteoporosis. The phytoestrogens, which are known to bind to the estrogenic receptor sites of the cell and trigger the components and processes of estrogenic activity, have a promising role in the treatment of osteoporosis.[7] The isoflavonoids are among the most active phytoestrogens in the flavonoid class. Ipriflavone, a synthetic flavonoid derivative,[8] was found to be effective in preserving bone mass in several models of experimental osteoporosis.[9] The isoflavones, found in soybeans, such as genistein, were found to prevent bone loss in the OVX rat model of osteoporosis.[10] Genistein was also found to suppress osteoclastic function, *in vitro* and *in vivo*.[11]

Punica granatum Linn. is one of the medicinal plants containing phytoestrogens. Many years, Punica juice known and use for traditional medicine such as dried pericarp and the juice of the fruits are employed as orally medication in the treatment of colic, colitis, leukorrhea, menorrhagia, oxyuriasis, paralysis, and external application to caked breast and to the nape of the neck in mumps and headache. Pomegranate juice is rich in antioxidants which general possess numerous important biological properties against cholesterol oxidation, protection against atherogenesis, anti-inflammatory, anti-aging, and protection against Alzheimer's disease and diabetes. However, the estrogenic effect of phytoestrogen as a selective estrogen receptors modulator from *P. granatum* has not been investigated, especially the tannin compound of pomegranates. Therefore, there is a great interest to investigate the effect and action of tannins of pomegranate on bone and reproductive tissue on osteoporosis model rat.[12]

MATERIALS AND METHODS

Chemicals and Reagents

Calcium, phosphorous, tartarent resistant acid phosphatase (TRAP), osteocalcin (OC), and alkaline phosphatase (ALP) kits were obtained from Erba Diagnostics, Mallaustr, Germany. Estrogen (Dr. Reddy's Laboratory, Hyderabad, India) and ketamine obtained from Neon Laboratories Ltd., Mumbai, India. All other chemicals and reagents were of analytical grade.

Plant Material and Preparation of Extract

The medicinal plant *P. granatum* seeds collected from the local market of Bengaluru, Karnataka. The collected plant authenticated by Prof. P.V. Laxminarayana, Botanist, and the herbarium specimen (SRMC025/2016-17) has been deposited in the pharmacology research laboratory. The 5 kg of plant material dried properly (naturally), coarse powdered and stored in a well-closed container in the dark. The plant material was extracted successively with petroleum ether, chloroform followed by ethanol in Soxhlet extractor. The ethanol extract was evaporated under reduced pressure to give an average yield of 35% w/w. The extract was then stored in a desiccator until the anti-osteoporotic activity studies were carried out.

Phytochemical Screening

Phytochemical screening of the ethanol extract of seeds of *P. granatum* was carried out by employing standard procedures and tests[13] to find out the presence of chemical constituents such as alkaloids, terpenoids, flavonoids, tannins, and coumarins.

Animals

Female Wistar albino rats weighing about 150–220 g in the age group of about 90 days were acclimatized to the experimental room at temperature 23±2°C, controlled humidity conditions (50–55%), and 12-h-light/12-h-dark cycle. Animals were caged with a maximum of two animals each in a polypropylene cage and were fed with standard food pellets (Hindustan Lever Ltd., India) and water *ad libitum*. The study was conducted after obtaining Ethical Committee Clearance from the Central Animal House of the Institutional Animal Ethics Committee of Sri Ramachandra College of Pharmacy, Porur, Chennai, India.

Acute Toxicity (OECD Guidelines 423)

Twenty healthy Wistar albino rats of either sex were randomly divided into two groups of equal size. Animals of both the groups were fasted overnight before the test. The first group was given 5000 mg/kg body weight of freshly prepared ethanol extract of *P. granatum* while the other group was given an equivolume of saline. The animals were examined at every 30 min up to a period of 3 h and then occasionally for an additional 4 h period; finally, 24 h mortality was recorded.

Experimental Protocol of Antiosteoporotic Activity

Experimental animals were divided randomly into six groups of six animals each. Group 1 was sham-operated and served as basal control. All the other groups were OVX and received treatment for 3 months, starting from the 15th day of ovariectomy. Group 2 received vehicle and served as OVX control. Group 3 was orally administered estrogen (2 mg/kg). Groups 4, 5, and 6 were orally treated with a suspension of ethanol extract of *P. granatum* in 5% tween 80 at three different doses of 100, 300, and 500 mg/kg body weight, respectively. After 7 days of acclimation, the rats were OVX. The rats were anesthetized with ketamine HCl (50 mg/kg, i.p), and ovaries were removed bilaterally. Sham-operated animals were performed in the same manner but only exposing the ovaries. They were administrated with prophylactic gentamicin (10 mg/kg, i.p) for 3 days.[14] The treatment of ethanol extract of *P. granatum* administered using oral gavage for 90 days. Body weight of all animals was measured weekly. [15] At the end of 90 days, all the rats were deprived of food for the whole night. On the next day, urine (0–24 h) was collected, then the animals were anesthetized by ketamine HCl (50 mg/kg, ip) and blood samples were withdrawn by retro-orbital puncture method. The blood samples were centrifuged at 2500 rpm for 15 min to separate the serum and preserved (−20°C) for analysis of serum calcium, phosphorus, ALP, tartarate resistant acid phosphatase (TRAP), and OC.[16] Immediately, after collecting the urine and blood samples, uterus was carefully removed and weighed. The lumbar vertebra and femurs were isolated and stored at −70°C until biochemical, biomechanical, and histopathological studies were performed.

Femur Physical Parameter

Fresh isolated left femurs were weighed using an electronic scale. The length of the femurs was measured using digital slide calipers. The length was measured from the proximal tip of the femur head to the distal tip of the medial condyle.[15] Bone volume and density were measured by the fluid displacement method.[17]

Lumbar Compression Test

The fourth lumbar vertebra was located and then it was isolated. The fresh vertebra was placed in digital hardness tester and compress until it got fractured and the reading was recorded in newtons (N).[15]

Femoral Ash Weight, Ash Percentage, and Ash Calcium

After measuring the bone length of the left femur, it was placed in tared fused silica crucibles and kept in Muffles furnace (Growell Instruments, Bengaluru, India), dried to a constant temperature at 600°C for 24 h. Then, ash weight was determined and the sample was suitably diluted with deionized water and assayed for calcium. [18]

Biochemical Estimation

The levels of serum calcium, phosphorous, ALP, TRAP, and OC, and urine calcium, inorganic phosphorus, and creatinine were measured using semi-automatic analyzer using commercially available test kits (Erba Diagnostics, Mallaustr, Germany).[15]

Histopathological Observation

The right femur was fixed in 10% formalin for 12 h at 4°C, decalcified in 5% ethylenediaminetetraacetic acid (EDTA) for 7 days and embedded in paraffin wax and cut into a sagittal plane section of 5 µm thickness of the femur. The sections were stained with hematoxylin and eosin and examined for histopathological changes under a light microscope.[19]

RESULTS

Effect of Ethanolic Extract of *Punica granatum* Seeds (EPGS) on Body Weight and Uterine Weight in OVX Rats

There was no significant change in body weight in all the groups. The ovariectomy resulted in a significant ($P < 0.001$) decrease in the uterine weight of OVX model group as compared to sham-operated group [Table 1]. Oral administration of 300 mg/kg and 500 mg/kg of *P. granatum* seeds extract showed significant increase ($P < 0.05$) and ($P < 0.001$) in uterine weight, respectively, and estrogen group showed weight gain as compared to OVX model group and this difference was statically significant ($P < 0.001$) after 3 months of treatment.

Effect of EPGS on Femur Physical Parameter in OVX Rats

The length and weight of the femur were decreased significantly ($P < 0.001$) in OVX model group as compared to sham-operated group [Table 2]. Following the administration of *P. granatum* seeds extract (100, 300, and 500 mg/kg) and estrogen in OVX rat's significant increase in length-weight and density was observed which was comparable to the sham-operated group. However, no significant changes in volume were observed in all the groups of animals. The dose-dependent protection in the bone loss was observed in *P. granatum* seeds extract-treated OVX rats.

The lumbar vertebra hardness was decreased significantly ($P < 0.001$) in OVX group as compared to sham-operated group [Table 2]. After treatment with *P. granatum* seeds

extract (100, 300, and 500 mg/kg) and estrogen in OVX rats, the significant increase in biomechanical strength was observed, which is comparable to sham group. The dose-dependent protection was observed in *P. granatum* seeds extract-treated OVX rats.

Effect of EPGS on Biochemical Parameters

The results of serum parameters in animals of different groups are shown in Table 3. The results indicate a reduction in the serum calcium ($P < 0.001$) and phosphorus ($P < 0.001$) in OVX model group. In contrast, urine calcium, creatinine, and phosphorous levels were significantly elevated. *P. granatum* seeds extract (100, 300, and 500 mg/kg) and estrogen groups showed [Table 4] significant ($P < 0.001$), dose-dependent recovery of serum calcium and phosphorus, whereas urine calcium, creatinine, and phosphorous levels were significantly ($P < 0.001$) decreased. Serum ALP and TRAP levels elevated significantly ($P < 0.001$) in OVX model group as compared to sham-operated group. In contrast, significantly ($P < 0.001$) reduction in levels of serum ALP levels was observed with *P. granatum* seeds extract (100, 300, and 500 mg/kg) and estrogen-treated groups. The OC

Table 1: Effect of EPGS on body weight and uterine weight in OVX rats

Treatment groups	Body weight (g)			Uterine weight (mg)
	Initial	Final	% changes in body weight	
Normal	160±15	172±2.16	7.5	625±3.854
Control (OVX) + saline	170±6.91	182±4.21	1.07	262±23.73^{c}
OVX + Estrogen (2 mg/kg)	180±15.41	176±13.47	3.0	598±11.08***
OVX + EPGS 100 mg/kg	160±8.93	178±6.50	11.25	253±8.946
OVX + EPGS 300 mg/kg	185±5.61	176±7.08	9.51	423±16.45***
OVX + EPGS 500 mg/kg	190±11.64	181±12.27	4.73	577±9.094***

All values are expressed as mean±SEM, n=6 analyzed by one-way analysis of variance (ANOVA) followed by multiple comparisons Dunnett's t-test, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ as comparison to sham-operated normal group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ as comparison to control group (OVX). EPGS: Ethanolic extract of *Punica granatum* seeds, OVX: Ovariectomized

Table 2: Effect of EPGS on femoral length, weight, volume, density, and fourth lumbar hardness in OVX rats

Treatment groups	Length (mm)	Weight (g)	Volume (ml)	Density (g/ml)	4th Lumbar hardness (kg/m) N
Normal	35.25±0.1297	0.543±0.115	0.337±0.00803	1.611±0.027	185±4.85
Control (OVX) +Saline	34.4±0.82	0.453±0.122	0.863±0.0092	0.562±0.0298^{c}	107±0.615^{c}
OVX + Estrogen (2 mg/kg)	35.7±0.277	0.535±0.0764	0.332±0.0172	1.611±0.0504***	188±0.919***
OVX + EPGS 100 mg/kg	33±0.26^{a}	0.522±0.0349	0.248±0.0212	2.10±24.7***	168±1.18^{c,***}
OVX + EPGS 300 mg/kg	34.3±0.368	0.565±0.0266	0.355±0.0765	1.59±0.055***	177±1.02^{a,***}
OVX + EPGS 500 mg/kg	36.2±0.696	0.527±0.0199	0.39±0.0511	1.35±0.0683***	177±0.816^{a,***}

All values are expressed as mean±SEM, n=6 analyzed by one-way analysis of variance (ANOVA) followed by multiple comparison Dunnett's t-test, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ as comparison to sham-operated normal group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ as comparison to control group (OVX). EPGS: Ethanolic extract of *Punica granatum* seeds, OVX: Ovariectomized

Table 3: Effect of EPGS on serum biochemical markers in OVX rats

Treatment groups	Serum				
	ALP (IU/L)	Calcium (mg/dl)	Phosphorous (mg/dl)	TRAP (mg/dl)	Osteocalcin (mg/dl)
Normal	89.02±0.4559	10.9±0.165	5.565±0.1049	1.25±0.032	0.512±0.0154
Control (OVX) + Saline	133.7±0.9655^{c}	7.44±0.117^{c}	3.560±0.0708^{c}	3.2±0.0223^{c}	0.269±0.0066^{c}
OVX + Estrogen 2 mg/kg	87.57±0.4853***	10±0.231^{b,***}	5.400±0.1262***	1.21±0.0343***	0.492±0.0502***
OVX + EPGS 100 mg/kg	105.1±0.5772***	7.91±0.17^{c}	4.217±0.0872^{c,***}	3.3±0.0737^{c}	0.3±00172^{c}
OVX + EPGS 300 mg/kg	97.86±0.4201^{***,c}	8.55±0.149^{c,***}	4.562±0.067^{c,***}	2.24±0.048^{c,***}	0.474±0.0191**
OVX + EPGS 500 mg/kg	84.26±0.9164^{***,c}	9.67±0.169^{c,***}	5.0±0.139^{b,***}	1.2±0.0462***	0.486±0.0027***

All values are expressed as mean±SEM, n=6 analyzed by one-way analysis of variance (ANOVA) followed by multiple comparison Dunnett's t-test, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ as comparison to sham-operated normal group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ as comparison to control group (OVX). EPGS: Ethanolic extract of *Punica granatum* seeds, OVX: Ovariectomized

level was significantly decreased in the OVX group when compared with other groups treated with standard and EPGS elevated the levels remarkably. Significantly increased level of creatinine, calcium, and phosphorous level was observed in the urine of EPGS treated rats [Table 4].

Effect of EPGS on Femoral Ash Weight, Ash Percentage, and Ash Calcium

The femur ash weight, ash percentage, and ash calcium were decreased significantly ($P < 0.001$) in the OVX model group as compared to sham-operated group [Table 5]. In contrast, weight of ash, percentage of ash, and calcium content were significantly increased in *P. granatum* seeds extract (100, 300, and 500 mg/kg) and estrogen-treated groups.

Histopathological Analysis

The femur section was examined for any histological changes [Figure 1]. The sham group animals showed normal architecture and normal bone compactness [Figure 1a]. The rats in the OVX model group showed fragility with disruptive, lytic changes, and thinning of the trabecula resulting in intertrabecular spaces widening [Figure 1b]. Estrogen and *P. granatum* seeds extract (100, 300, and 500 mg/kg) groups exhibited significant restorative progress with increased ossification, mineralization, and increased osteoclastic activity and reduced bone resorption, which indicates the recovery with essential features of normal bone [Figure 1c-f].

DISCUSSION

The aim of this study was to investigate the effects of the pomegranate seeds on bone protection and effects on reproductive organs. OVX rats are classically used as an animal model for studying the effect of postmenopausal bone loss. Furthermore, they may provide a useful model for investigating the biological effect of *Punica granatum* Linn on bone loss in OVX rats. Pomegranate from ethanol of extract contains tannins especially ellagic acid.[17,18]

The pomegranate seed contains various active metabolites and these compounds include tannins as ellagic acid, gallotannic acid, alkaloids, glycosides, phenols, saponins, coumarins, flavones, and resins. The previous study showed that ellagic acid prevents bone loss by increasing mineralization of bone through osteoblast. The present study was investigated the potential preventive effects of tannin-content ethanolic extract of *P. granatum* L. which contains ellagic acid to prevent bone loss in an animal model of osteoporosis. The administration of the ethanol extract of *P. granatum* (L) seeds prevented OVX-induced increase in average body weight gain in rats. These results also support by the previous study that compounds that prevented OVX-induced uterine atrophy and increases in

Table 4: Effect of EPGS on urinary biochemical markers in OVX rats

Treatment groups	Urine		
	Creatinine (mg/dl)	Calcium (mg/dl)	Phosphorous (mg/dl)
Normal	1.5±0.0194	0.443±0.0369	0.443±0.03686
Control (OVX) + Saline	0.467±0.0119^{c}	0.258±0.011^{b}	0.258±0.01101^{b}
OVX + Estrogen 2 mg/kg	1.25±0.204***	0.401±0.0519*	0.4005±0.05188*
OVX + EPGS 100 mg/kg	0.611±0.204^{c}	0.33±0.00801	0.3300±0.008012
OVX + EPGS 300 mg/kg	1.21±0.0291***	0.405±0.0534*	0.4050±0.05338*
OVX + EPGS 500 mg/kg	1.35±0.0263***	0.470±0.8421***	0.4706±0.00842**

All values are expressed as mean±SEM, n=6 analyzed by one-way analysis of variance (ANOVA) followed by multiple comparison Dunnett's t-test, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ as comparison to sham-operated normal group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ as comparison to control group (OVX). EPGS: Ethanolic extract of *Punica granatum* seeds, OVX: Ovariectomized

Table 5: Effect of EPGS on ash content of femoral bone in OVX rats

Treatment groups	Ash weight (g)	Ash (%)	Calcium (mg/dl)
Normal	0.59±0.0146	60.83±1.815	9.42±0.163
Control (OVX) +Saline	0.380.00894^{c}	38.00±0.8944^{c}	6.25±0.066^{c}
OVX + Estrogen 2 mg/kg	0.5983±0.02040***	62.17±1.352***	10.4±0.106***
OVX + EPGS 100 mg/kg	0.435±0.005**	42.83±0.4773**	7.19±0.0254***
OVX + EPGS 300 mg/kg	0.530±0.0073***	53.00±0.7303***	8.52±0.138***
OVX + EPGS 500 mg/kg	0.553±0.00882***	55.50±0.8466***	9.49±0.104***

All values are expressed as mean±SEM, n=6 analyzed by one-way analysis of variance (ANOVA) followed by multiple comparison Dunnett's t-test, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ as comparison to sham-operated normal group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ as comparison to control group (OVX). EPGS: Ethanolic extract of *Punica granatum* seeds, OVX: Ovariectomized

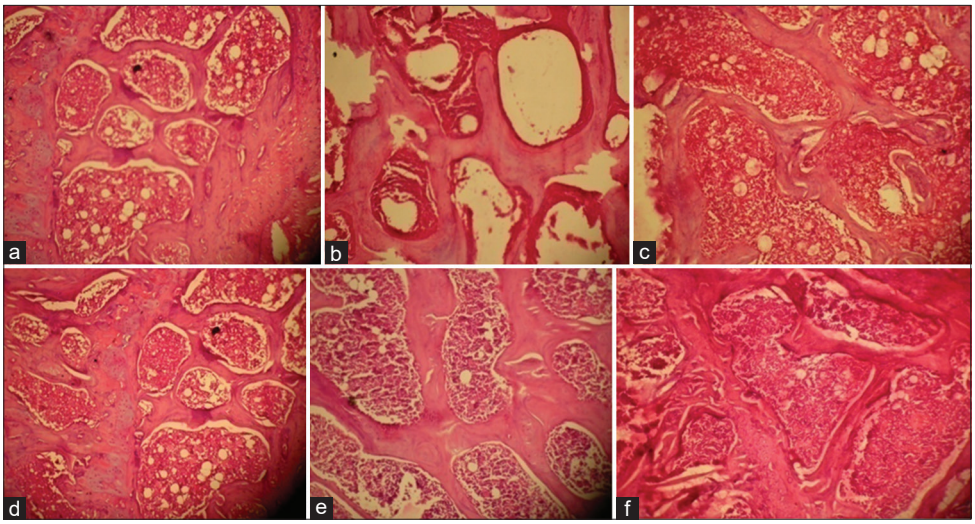

Figure 1: Histopathological studies. (a) Effect of 5% Span 20 on sham-operated normal femur histopathology: Photomicrograph of the epiphyseal region of sham-operated normal showing normal, compact, and uniform trabecula with intertrabecular spaces. (b) Effect of 5% Tween 80 on control (ovariectomized [OVX]) femur histopathology: Photomicrograph of the epiphyseal femoral region showing sparse, thinning of trabecula, loss of interconnectivity and widening of intertrabecular spaces, trabecula markedly disruptive, lytic changes, and decrease in cells due to bone resorption. (c) Effect of estrogen on OVX rats femur histopathology: Photomicrograph of the epiphyseal femoral region showing moderately thick elongated trabecula and narrowed, last intertrabecular spaces and showing the restoration of normal architecture and markedly diminished bone resorption by increasing bone cells. (d) Effect of the ethanolic extract of *Punica granatum* seeds (EPGS) 100 mg/kg on OVX rats femur histopathology: Photomicrograph of the epiphyseal femoral region showing moderately, thick elongated trabecula and narrowed intertrabecular space, and restoration of normal architecture along with increasing bone cells. (e) Effect of EPGS 300 mg/kg on OVX rats femur histopathology: Photomicrograph of the epiphyseal femoral region showing moderately thick elongated trabecula, narrowed and compact intertrabecular spaces associated with increased osteoblast cells. (f) Effect of EPGS 500 mg/kg on OVX rats femur histopathology: Photomicrograph of the epiphyseal femoral region showing finely thick, elongated trabecula, and narrowed, last intertrabecular spaces showing complete restoration of normal architecture by forming cells

body weight gain, serum TRAP, OC, phosphorous and urine creatinine, calcium, and phosphorous while a significant decrease in ALP and calcium levels in serum. According to the previous report, rats in the OVX group had lower densities of the right femur and tibiae because of reducing the ovariectomy-induced increase in bone resorption. The administration of the ethanolic extracts of *P. granatum* (L) 100, 300, and 500 mg/kg effectively prevented OVX-induced lowering the bone density. These observations are supported by the previous study that ellagic acid significantly prevented bone loss in OVX rats by increasing the mineralization of bone.[19]

Estrogen-deficient OVX osteoporosis animal models have been used to evaluate osteoporotic drugs. Serum OC levels are generally considered to be a marker of bone turnover and serum ALP levels to be a marker of bone formation. As the progression of OVX-related osteoporosis, serum OC levels were generally increased along with the increases of bone turn over, but serum ALP contents were decreased along with the inhibition of bone formations. On the contrary, the serum OC levels were decreased, but serum ALP levels were increased in the present study, which suggested that the treatment activated the osteoblast differentiation and inhibited the bone mineralization and turn over. Bone mineral density provides information regarding the efficacy of anti-osteoporotic agents. Microscopic observations of bones also provide valuable information regarding bone morphology. In osteoporotic animals, the histological profiles are changed compared with sham controls regardless of the cause, especially in the trabecular and cortical bones. The efficacy of various anti-osteoporotic agents has been evaluated using bone histology, namely, some histomorphometrical indices for bone mass and bone formation are decreased concomitantly with increased bone resorption and this data can help predict the efficacy of anti-osteoporotic agents.[20]

Many studies have shown that pomegranate juice and pomegranate polyphenol extracts can prevent many types of cancer, cardiovascular disease, diabetes, Alzheimer's disease, arthritis, and colitis. Recent reports have shown that pomegranate seed oil and pomegranate juice contain several species of flavonoids and anthocyanidins and that their antioxidant activity is 3 times more potent than that in red wine or green tea extract. The estrogenic activity of the ethanolic extract of *P. granatum* seeds is mainly due to flavones and alkaloids, to a lesser extent, gallotannic acid. Flavones have various biological activities and can improve metabolic symptoms and bone-protective effects in menopause. Flavonoids exert a weak estrogen-like effect by binding to ER-α and -β in various tissues. Furthermore, flavonoids have the ability to interact with estrogen receptors and to control the activity of CYP19, an important enzyme in estrogen biosynthesis, and/or steroid

dehydrogenases (e.g., 11β-hydroxysteroid dehydrogenase). These effects induce various alterations causing a change in the overall hormonal balance, resulting in protection against bone loss and reducing osteoporotic effects and other menopausal symptoms. Ethanolic extract of *P. granatum* seeds on postmenopausal symptoms is mainly due to the phytoestrogenic effects of flavonoids, gallotannic acid, and alkaloids.[20] The ethanolic extract of *P. granatum* seeds relieves climacteric symptoms through anti-osteoporotic activity.

CONCLUSIONS

Collectively, our results suggest that a potential effect of the ethanolic extract of *P. granatum* seeds increased the anti-osteoporotic effects in OVX rats. Therefore, we suggest that pomegranate seeds are promising new potent protective agents for relieving osteoporosis in menopausal females. Further studies are needed to investigate the efficacy of the ellagic acid in humans.

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REFERENCES

1. Kelly PJ. Is osteoporosis a genetically determined disease? Br J Obstet Gynaecol 1996;103 Suppl 13:20-6.
2. Arjmandi BH, Alekel L, Hollis BW, Amin D, Stacewicz-Sapuntzakis M, Guo P, *et al*. Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. J Nutr 1996;126:161-7.
3. Genant HK, Baylink DJ, Gallagher JC. Estrogens in the prevention of osteoporosis in postmenopausal women. Am J Obstet Gynecol 1998;161:1841-6.
4. Gruber HE, Ivey JL, Baylink DJ, Matthews M, Nelp WB, Sisom K, *et al*. Long-term calcitonin therapy in postmenopausal osteoporosis. Metabolism 1984;33:295-303.
5. Shankar BP. Osteoporosis-a silent epidemic. Pharm Times 2002;34:21-2.
6. Mitra SK, Venkataranganna MV, Udupa UV, Gopumadhavan S, Seshadri SJ, Rafiq M, *et al*. The beneficial effect of OST-6 (OsteoCare), a herbomineral formulation, in experimental osteoporosis. Phytomedicine 2001;8:195-201.
7. Adams NR. Phyto-oestrogens. In: Cheeke P, editor. Toxicants of Plant Origin. Boca Raton, FL: CRC Press; 1989. p. 23-51.
8. Agnusdei D, Zacchei F, Bigazzi S, Cepollaro C, Nardi P, Montagnani M, *et al*. Metabolic and clinical effects of ipriflavone in established post-menopausal osteoporosis. Drugs Exp Clin Res 1989;15:97-104.
9. Benvenuti S, Tanini A, Frediani U, Bianchi S, Masi L, Casano R, *et al*. Effects of ipriflavone and its metabolites on a clonal osteoblastic cell line. J Bone Miner Res 1991;6:987-96.
10. Fanti P, Monier-Faugere MC, Geng Z, Schmidt J, Morris PE, Cohen D, *et al*. The phytoestrogen genistein reduces bone loss in short-term ovariectomized rats. Osteoporos Int 1998;8:274-81.
11. Blair HC. Action of Genistein and Other Tyrosine Kinase Inhibitor in Preventing Osteoporosis. Brussels, Belgium: Second International Symposium on the Role of Soya in Preventing and Treating Chronic Disease; 1996. p. 15-8.
12. Sreeja S, Santhosh Kumar TR, Lakshmi BS, Sreeja S. Pomegranate extract demonstrate a selective estrogen receptor modulator profile in human tumor cell lines and *in vivo* models of estrogen deprivation. J Nutr Biochem 2012;23:725-32.
13. Trease GE, Evans WC. Pharmacognosy. London: Harcourt publisher; 1979.
14. Lasota A, Danowska-Klonowska D. Experimental osteoporosis--different methods of ovariectomy in female white rats. Rocz Akad Med Bialymst 2004;49 Suppl 1:129-31.
15. Reddy NP, Lakshmana M, Udupa UV. Antiosteoporotic activity of OST-6(Osteocare), a herbomineral preparation in calcium deficient ovariectomized rats. Phytother Res 2004;18:25-9.
16. Shirwaikar A, Khan S, Malini S. Antiosteoporotic effect of ethanol extract of *Cissus quadrangularis* Linn. on ovariectomized rat. J Ethnopharmacol 2003;89:245-50.
17. Das AS, Das D, Mukherjee M, Mukherjee S, Mitra C. Phytoestrogenic effects of black tea extract (*Camellia sinensis*) in an oophorectomized rat (*Rattus norvegicus*) model of osteoporosis. Life Sci 2005;77:3049-57.
18. Wang J, Shang F, Mei Q, Wang J, Zhang R, Wang S. NO-donating genistein prodrug alleviates bone loss in ovariectomised rats. Swiss Med Wkly 2008;138:602-7.
19. Sepulveda L, Ascacio A, Rodríguez-Herrera R, Aguilera-Carbo A, Aguilar CN. Ellagic acid: Biological properties and biotechnological development for production processes. Afr J Biotechnol 2011;10:4518-23.
20. Bahtiar A, Sunarmi A, Amalia R, Nurul Q, Puspita EW, Ade A. Polar Fraction of L. peel extract *Punica granatum* increased osteoblast number on ovariectomized rat bone. Int J Herb Med 2014;2:65-70.

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