Role of some phytoestrogens in recovering bone loss: histological results from experimental ovariectomized rat models

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Background/aim

Osteoporosis is a disease characterized by a decrease in bone mass and is widely recognized as a major health problem. Ovarian hormone deficiency is a major risk factor for osteoporosis. A sharp decrease in ovarian estrogen production is the predominant cause of rapid bone loss and deterioration of bone architecture, resulting in increased bone fragility during the first decade after menopause.

Materials and methods

A total of 70 albino rats were used, divided into seven groups of 10 rats each. Group 1 was subjected to sham operation and used as a control group. In group 2, rats were ovariectomized (OVX) and used as a model of postmenopausal osteoporosis. Three months after the operation the OVX rats (group 2) were divided into six subgroups: one was considered the positive control group; another one was treated with synthetic estrogen compound; and the other four subgroups were fed a diet containing red clover, fennel, carob, and a combination of the three plants. At the end of the experiment (after 3 months' treatment) the animals were killed, and the femur shafts were extracted, decalcified, and processed into paraffin blocks. Sections were stained with hematoxylin and eosin for histopathological, image analysis, and morphometric studies. Other sections were stained with periodic acid Schiff for histochemical investigations.

Results

The histopathological results of this study revealed that ovariectomy caused a decrease in thickness of the cortical compact bone in the middle shaft of the femur and of the trabeculae in cancellous bone in the head of the femur bone. Histochemical results showed new bone formation in sections of rats treated with plants. The best results were detected in sections of rats treated with a combination of the three plants. Red clover, fennel, and carob individually or combined have a better ameliorating effect on ovariectomy-induced osteoporosis than does synthetic estrogen compound.

Conclusion

Treatment of OVX rats with phytoestrogens such as red clover, fennel, and carob might improve the histopathological and histochemical changes and morphometric parameters in bone with ovariectomy-induced osteoporosis.

Keywords:
carob, estrogen, fennel, osteoporosis, ovariectomy, rat, red clover

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Introduction

Osteoporosis (OP) is a bone metabolic disease characterized by low bone mineral density (BMD) with high risk for fractures. It occurs when there is an imbalance between bone resorption and bone formation during the bone remodeling process [1]. OP is a silent epidemic problem; it has now become a major health hazard affecting over 2000 million people worldwide [2]. OP is generally viewed as resulting from a combination of age-related, hormonal, dietary, lifestyle, and genetic factors, all of which can lead to reduced bone mass [3].

Ovariectomy provides the most popular model for studying events associated with postmenopausal osteoporosis (PMO) with estrogen deficiency, and it has been well established that ovariectomy elicits bone loss and increases bone turnover in rats. Ovariectomized (OVX) rats are widely accepted models for PMO [4]. The most common type of OP is bone loss associated with ovarian hormone deficiency at menopause [5], which leads to loss of bone mass [6]. Women are generally affected four times more than men, and fracture rates among women are approximately twice as high as that of men [7].

Estrogen deficiency has been regarded as a critical cause of OP, which can result from naturally or surgically induced menopause [8]. Reduction in estrogen leads to increased osteoclastic activity, which enhances bone loss by stimulating bone resorption because of reduced hormonal control over osteoblast cell activity [9]. The osteoblasts and osteoclasts have estrogen receptors, and estrogen affects bones partly because of these receptors. It has been proposed that estrogen causes depletion in the number of osteoclasts in bone by inhibiting maturation at the cellular level while enhancing the synthesis of cytokines that play roles in bone formation [10].

Loss of estrogens accelerates the effects of aging on bone by decreasing the defense against oxidative stress. Both aging and loss of sex steroids have adverse effects on skeletal homeostasis [11]. Lean *et al.* [12] noted that estrogen exerts beneficial actions through suppression of reactive oxygen species (ROS) that stimulate osteoclasts, the cells that resorb bone. Thus, estrogen might prevent bone loss by enhancing oxidant defenses in bone. They also found that estrogen deficiency after ovariectomy causes bone loss by lowering thiol antioxidants in osteoclasts. This directly sensitizes osteoclasts to osteoclastogenic signals and leads to ROS-enhanced expresion of cytokines that promote osteoclastic bone resorption.

Treatment with natural herbs is likely to cause fewer side effects compared with the presently used synthetic drugs [13].

Phytoestrogens appear to offer the most potential for the prevention of bone loss and have attracted attention as a possible agent in preventing and treating PMO, preventing cancer, and relieving menopausal symptoms [14].

Phytoestrogens are similar to mammalian estrogens both structurally and functionally [15]. There is evidence that diets containing high levels of phytoestrogenic isoflavones are associated with a low incidence of OP and menopausal symptoms [5].

Red clover (*Trifolium pratense*) supplementation has been the subject of much interest for its role in the reduction of menopausal symptoms and conditions related to aging because of their high concentrations of phytoestrogens [16]. It contains four important estrogenic isoflavones (daidzein, genistein, formononetin, and biochanin A) and coumestans [17]. Red clover isoflavones (RCI) are being increasingly used in dietary supplements for their purported estrogenic effect in in-vivo and *in-vitro* assays [18]. Long-term administration of isoflavones was found to positively affect bone metabolism [19]. The positive effect of isoflavones on bone metabolism may be mediated by at least two mechanisms: the first is the impact on osteoclasts through activation of apoptosis, and the second is the inhibition of tyrosine kinase activity through modulation of membrane endoplasmic reticulum (ER) with consecutive changes in the activity of alkaline phosphatase [20].

Fennel (*Foeniculum vulgare* Mill; *Apiaceae* family) is one of the most widespread annual or perennial plants with an aromatic odor. The most frequently investigated is the essential oil, which has shown antioxidant, antimicrobial, and hepatoprotective activity. Many herbs are well known to contain large amounts of phenolic antioxidants, which are mainly composed of phenolic acids and flavonoids [21].

Carob (*Ceratonia siliqua* L.; *Leguminosae* family) contains a remarkable amount of condensed tannins and other polyphenols [22]. Carob polyphenols protect against decreased lipid peroxidation induced by cisplatin administration [23]. The aqueous extract of carob induces a depletion of hydrogen peroxide in the kidney, liver, and brain but not in heart tissues [24]. Hydrogen peroxide is an important ROS because of its ability to penetrate biological membranes. However, it may be more toxic if converted to hydroxyl radicals in the cell, leading to lipid peroxidation [25] and oxidative DNA damage [26].

Therefore, the aim of this study was to evaluate the effectiveness of red clover, fennel, carob, and estrogen on the progression of bone loss (OP) in OVX female rats.

Materials and methods

Experimental animals

Seventy female Sprague–Dawley rats, aged 7 weeks and weighing 170–190 g at the beginning of the experiment, were used in this study. They were housed two per cage and maintained in standard conditions under a 12 : 12 light/dark cycle, at a temperature of 22 ± 1°C and 55–60% relative humidity. Rats were fed a standard nutritionally balanced diet according to AIN-93 [27] and drinking water *ad libitum*.

After a 7-day adaptation period to the controlled laboratory conditions, the animals were randomly divided into seven groups of 10 rats each.

The first group: in this group female rats (n = 10) were anesthetized with diethyl ether and subjected to sham operation.

The second group: in this group the ovaries of the remaining female rats (n = 60) were removed bilaterally according to the method described by Waynforth [28] and Lasota and Danowska-Klonowska [29]. Three months after the operation, this group was subdivided into:

2.1 (C-OVX): OVX control rats fed a standard diet devoid of any additives.
2.2 (ER-OVX): OVX rats treated with estrogen synthetic compound at a dose of 50 μg/kg/day.
2.3 (RC-OVX): OVX rats fed a diet containing 4% red clover.
2.4 (FE-OVX): OVX rats fed a diet containing 7.68% fennel.
2.5 (CA-OVX): OVX rats fed a diet containing 0.46% carob.
2.6 (COM-OVX): OVX rats fed a diet containing a mixture of all additives.

The OVX rats were subjected to the following treatments for 3 months.

Histopathological examination

At the end of the experimental period, the animals were killed and the shafts of the femur were removed, immersed in glutaraldehyde, and after 4 h were decalcified with EDTA solution for 20 days. Paraffin (5 μm) tissue sections from the middle shaft of the femur were cut using a conventional technique. Sections were stained with hematoxylin and eosin [30] and examined under a light microscope.

Morphometric study

The morphometric analysis was performed at the Pathology Department, National Research Center, using a Leica Qwin 500 Image Analyzer (Leica Imaging Systems Ltd, Cambridge, UK).

To measure the mean thickness of the outer cortical bone of the middle shaft of the femur, perpendicular lines were drawn from the periosteum to the endosteum at many sites [31].

Morphometric analysis is carried out on routine hematoxylin and eosin-stained slides. We count the maximum number of osteocytes in a frame area of 3905.5 μm^2 at a magnification of ×100. The results appear automatically on the monitor in the form of the distance measured in μm and area in μm^2 with the mean, SD, the minimum length, and the maximum length and area measured.

Histochemical studies

Staining with periodic acid Schiff (PAS) for demonstration of newly formed bone tissue [32] was performed on sections of the left femur bone of rats in all groups.

Areas of reactivity were marked and the optical density of PAS was measured using the gray image menu in 10 small measuring frames in each specimen. The image was transformed into a gray image (a grid of pixels), each representing the intensity of brightness at that point by a range of numbers.

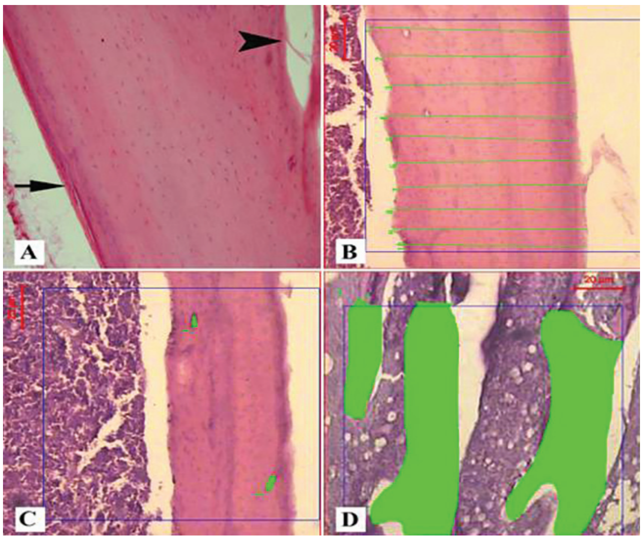
The experiment was conducted in accordance with the National Regulations of Animal Welfare and Institutional Animal Ethical Committee (IAEC), National Research Centre.

Results

Histological and morphometric results

Histological sections of the middle shaft of femur from rats of the sham-operated control group showed that bone tissue was of the compact type, covered by two layers, the periosteum located externally, which is a dense connective tissue, and the endosteum, a thin cell-rich connective tissue, lining the internal surface of the bone facing the bone marrow cavity. Within the bone matrix, osteocytes in their lacunae were detected (Fig. 1a).

Figure 1



Longitudinal sections of the middle shaft of femur of (a) a sham-operated rat shows an oute fibrous layer, periosteum (arrow), and an inner layer facing the marrow cavity, endosteum (arrowhead). (b) The same group shows normal cortical width of the shaft. (c) A sham-operated rat showing normal osteocytes and Haversian canals. (d) The head of the femur of a sham-operated rat showing normal architecture of the trabeculae of the inner cancellous bone and bone marrow spaces. (a) Hematoxylin and eosin-stained section; (b–d binary image morphometric measurement; ×400.

The head of the femur bone was formed of cancellous bone. Trabeculae of the cancellous bone of control rats were composed of a network of irregular bone lamellae between which osteocytes resided in their lacunae. Bone marrow spaces were observed between trabeculae (Fig. 1b).

In the case of OVX rats, examination of the middle shaft of the femur showed marked decrease in the thickness of the compact bone of the shaft (49.01 ± 12.02) as compared with the sham-operated group (78.43 ± 5.33) and decrease in the number of osteocytes (26 vs. 79). Many osteoporotic cavities, resorption cavities, and calcified cartilage were observed in bone tissue. Erosion cavities were detected on the outer surface (Fig. 2a and Table 1). An increase in the mean areas of Haversian canals was observed (21.15 ± 15.69) as compared with the sham-operated group (15.34 ± 9.97) (Fig. 2b and Table 1).

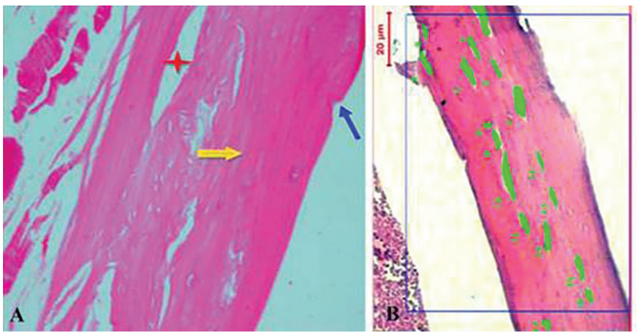
Examination of cancellous bone in the head of the femur revealed decreased mean areas of bone trabeculae (276.08 μm^2) as compared with the sham-operated group (1220.9 μm^2) (Table 1). Also, widening of bone marrow spaces and increase in blood vascularity within the bone marrow were noticed (Fig. 3).

Examination of bone sections of the middle shaft in OVX rats treated with synthetic estrogen compound showed slight increase in the thickness of the shaft cortical bone (52.52 ± 1.4 μm), compared with the OVX group (49.01 ± 12.02). The number of osteocytes was also slightly increased (n = 47), as compared with OVX rats (n = 26). Decrease in the mean areas of Haversian canals (16.74 ± 6.45 μm^2) was observed compared with the OVX group (21.15 ± 15.69 μm^2). Many osteoporotic cavities were still present. Irregular erosion of endosteal surface could be observed (Fig. 4a and Table 1).

In the head of the femur of this group, decreased mean areas of bone trabeculae (329.11 μm^2) were observed compared with the sham-operated group (1220 μm^2) (Table 1 and Fig. 4b). However, slight increase in mean areas of bone trabeculae (329.11 μm^2) was seen compared with OVX rats (276.08 μm^2).

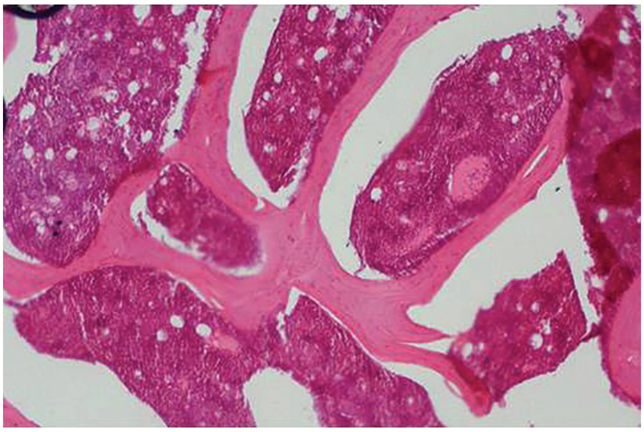
In the case of OVX rats treated with red clover, an improvement was seen in pathological changes in the form of increase in cortical bone thickness of the middle shaft (61.72 ± 1.9 μm) as compared with the OVX

Figure 2



Longitudinal sections of the middle shaft of (a) an overiectomized rat showing many osteoporotic cavities (star), deformation of the general architecture of the tissue, and erosion cavities on the endosteal surface (blue arrow). (b) An overiectomized rat showing widened Haversian canals. (a) Hematoxylin and eosin-stained section; (b) binary image morphometric measurement; ×400.

Figure 3



A photomicrograph of a section of trabecular bone of an ovariectomized rat showing marked reduction in trabecular thickness and widening of bone marrow spaces. Hematoxylin and eosin-stained section, ×400.

Table 1 Mean area of cortical bone thickness (shaft), mean Haversian canals' area, number of osteocytes and trabecular thickness of different groups

Groups	Cortical bone thickness (μm)	Haversian canal area (μm^2)	Number of osteocytes	Trabecular mean area (μm^2)
C negative	78.43 ± 5.33	15.34 ± 9.97	79	1220.90
Ovarectomized	49.01 ± 12.02	21.15 ± 15.69	26	276.08
Estrogen	52.52 ± 1.42	16.74 ± 6.45	47	329.11
Red clover	61.72 ± 1.9	15.34 ± 9.97	79	763.25
Fennel	64.62 ± 3.54	28.22 ± 20.77	36	351.65
Carob	89.00 ± 3.23	17.39 ± 10.51	34	913.25
Combination	100.93 ± 4.59	8.70 ± 3.48	42	1850.97

group (49.01 ± 12.02 μm), and the number of osteocytes returned to normal (n = 79). However, erosion cavities were still noticed on both sides of the cortical bone (Fig. 5a and Table 1). There was a decrease in the mean area of Haversian canals (15.34 ± 9.97) as compared with the OVX group (21.15 ± 15.69) (Table 1).

Decreased mean area of bone trabeculae of the femur head cancellous bone (763.25 μm^2) was observed compared with the sham-operated group (1220.9 μm^2), whereas the mean area demonstrated an increase compared with OVX rats (267.08 μm^2) (Table 1 and Fig. 5b).

OVX rats treated with fennel revealed an improvement in histological changes. Examination of bone sections of the middle shaft of the femur showed increase in thickness of the cortical shaft bone (64.62 ± 3.54 μm) as compared with OVX rats (49.01 ± 12.02 μm), although it was nonuniform, and an increased number of osteocytes (n = 36 vs. 26 in OVX rats). Irregularity was detected on the endosteal surface (Table 1 and Fig. 6a).

Cancellous bone of the head of the femur showed moderate increase in the mean areas of the bone trabecular surface (351.65 μm^2) as compared with OVX rats (276.08 μm^2) (Table 1 and Fig. 6b).

Examination of the compact bone of the middle shaft of the femur of OVX rats treated with carob showed an increase in shaft cortical thickness (89.00 ± 3.23 μm) compared with untreated OVX rats(49.01 ± 12.02 μm), and an increased number of osteocytes (34 vs. 26) denoting recovery of bone tissue. The mean areas of Haversian canals were smaller in the OVX group fed carob (17.39 ± 10.51 μm^2) compared with untreated OVX rats (21.15 ± 15.69 μm^2) (Table 1 and Fig. 7a).

Marked increase in mean areas of cancellous bone trabeculae (913.25 μm^2) was observed in this group compared with untreated OVX rats (276.08 μm^2) (Table 1 and Fig. 7b).

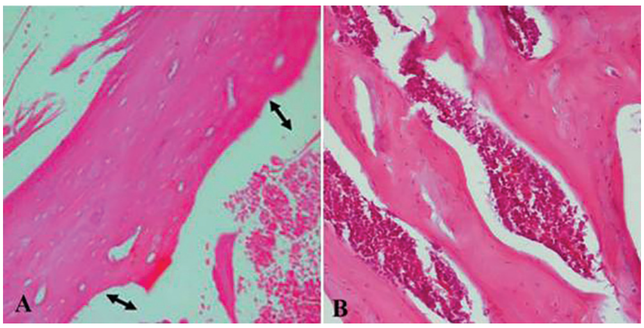
Examination of bone sections of the middle shaft of the femur in OVX rats treated with a combination of red clover, fennel, and carob showed an improvement in the pathological changes in the form of increased thickness of the cortical shaft bone (100.93 ± 4.59 μm) compared with untreated OVX rats (49.01 ± 12.02 μm), as well as increased number of osteocytes (n = 42 vs. 26) and narrowing in the mean area of Haversian canals (8.70 ± 3.48 vs. 21.15 ± 15.69) (Table 1 and Fig. 8a).

By examining the head of the femur of this group, increase in mean areas of cancellous bone trabeculae (1850 μm^2) was observed compared with the sham-operated group (1220.90 μm^2) and the untreated OVX group (276.08 μm^2) (Table 1 and Fig. 8b).

Histochemical results

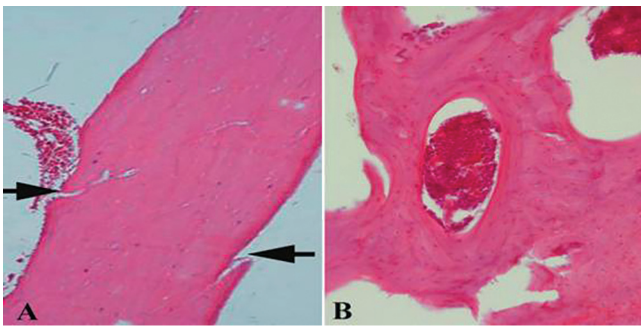
Sections of bone in the sham-operated group showed PAS-stained areas in cement lines in the shaft compact bone (Fig. 9a) and trabeculae (Fig. 9b). In the case of OVX rats cement lines were unapparent in PAS-stained sections in the shaft and in bone trabeculae (Fig. 10a and b). The gray level of PAS staining of this group was markedly reduced when compared with the sham-operated group (0.07 vs. 0.3) (Fig. 16).

Figure 4



(a) A longitudinal section of cortical bone tissue of the middle shaft of the femur of an ovariectomized rat treated with estrogen synthetic compound showing decrease in thickness in the shaft as compared with sham-operated rats; resorption cavities are still present (arrows) and an increase in the number of osteocytes is evident. (b) A section of trabecular bone of the same group showing mild increase in trabecular thickness as compared with the ovariectomized group. Hematoxylin and eosin-stained section, (a) ×200 and (b) ×400.

Figure 5



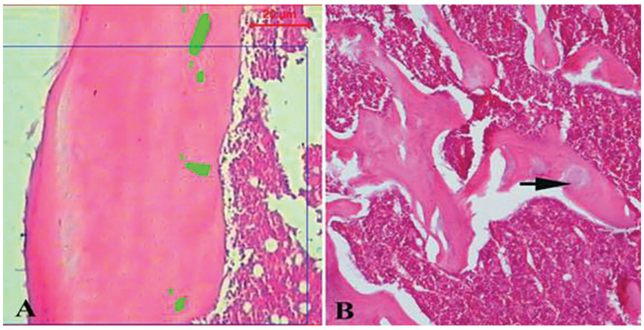
(a) A longitudinal section of the middle shaft of the femur of an ovariectomized rat fed red clover showing mild increase in cortical bone thickness, although erosion cavities are observed on both sides of the bone (arrows). (b) A section of trabecular bone of the same group showing noticeable increase in trabecular area with increase in the number of osteocytes. Hematoxylin and eosin-stained section, (a) ×200 and (b) ×400.

The bone of OVX rats treated with synthetic estrogen compound exhibited faint PAS reaction in cement lines in both cortical and trabecular bone (Fig. 11a and b). The gray level of the staining of this group showed slight increase when compared with OVX (0.1 vs. 0.07) (Fig. 16). Bone sections in the OVX group treated with red clover exhibited an intense PAS-positive reaction in cement lines (Fig. 12a and b). The gray level of staining in OVX rats treated with red clover returned to normal levels (0.3) (Fig. 16). The bone of OVX rats treated with fennel showed distinct PAS-positive reaction in cement lines of the shaft and bone trabeculae (Fig. 13a and b), whereas sections from OVX rats fed carob showed moderate PAS-positive reaction in the cement lines of shaft and bone trabeculae (Fig. 14a and b). The gray levels of staining in these two groups were nearly normal (0.33 and 0.25, respectively) (Fig. 16). Examination of bone sections of OVX rats treated with a combination of red clover, fennel, and carob exhibited very intense distinct PAS-positive reaction in the cement lines in the shaft and trabeculae (Fig. 15a and b), which was markedly increased when compared with OVX rats (0.4 vs. 0.07) and the sham-operated group (0.4 vs. 0.3) (Fig. 16).

Discussion

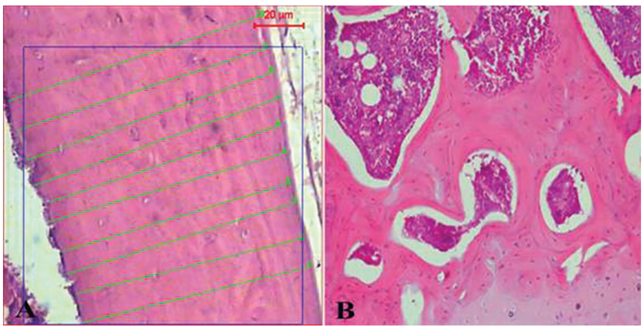
OP is characterized by decrease in bone mass and is widely recognized as a major health problem. It is a multifactorial process and is associated with demographic and lifestyle factors, morbidity, drug use, medical history, and altered hormonal profile [33]. Worldwide, OP is considered second only to cardiovascular disease as a leading health problem [34]. Estrogens play an important role in skeletal homeostasis. A sharp decrease in ovarian

Figure 6



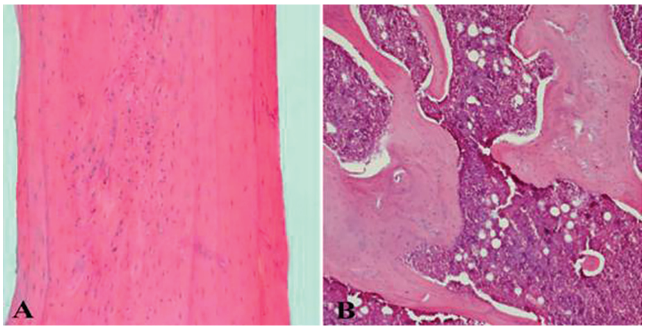
(a) A longitudinal section of the middle shaft of the femur of an ovariectomized rat fed fennel showing nonuniform increase in thickness of the cortical bone with mild reduction in the area of Haversian canals. Irregularity of endosteal surface was observed. (b) A section of trabecular bone of the same group showing moderate increase in trabecular areas and calcified cartilage (arrow). (a) Binary image morphometric measurement; (b) hematoxylin and eosin-stained section; ×400.

Figure 8



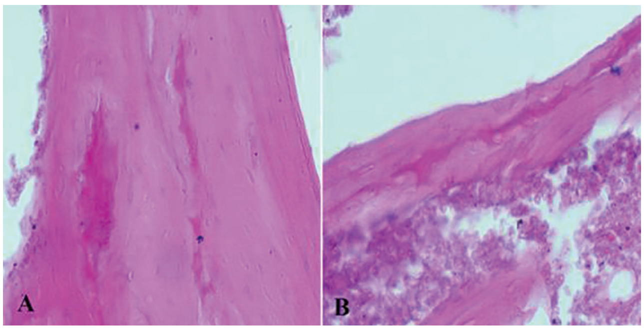
(a) A longitudinal section of the middle shaft of the femur of an ovariectomized rat fed a combination of the three plants showing normalization of the cortical bone thickness the numbers shown in the figure are the numbers of measuring lines used by image analyzer system. (b) A section of trabecular bone of the same group shows considerable amelioration of trabecular thickness. (a) Binary image morphometric measurement, (b) hematoxylin and eosin-stained section; ×400.

Figure 7



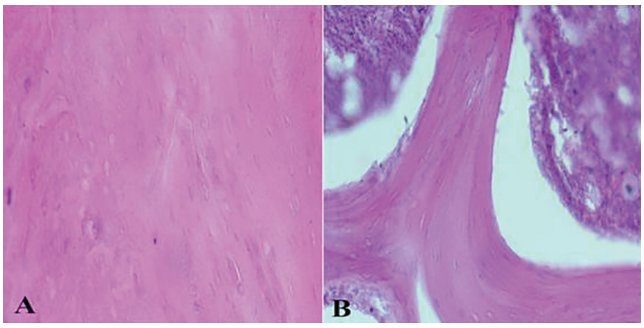
(a) A longitudinal section of the middle shaft of the femur of an ovariectomized rat fed carob showing marked increase in cortical bone thickness with increased number of osteocytes. No erosion or osteoporotic cavities are observed. (b) A section of trabecular bone showing marked increase in trabecular area. Hematoxylin and eosin-stained section, ×400.

Figure 9



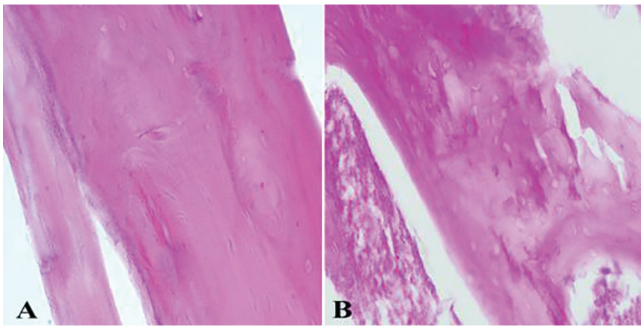
A longitudinal section of cortical bone of the middle shaft of femur (a) and trabecular bone (b) of sham-operated rats showing periodic acid Schiff (PAS)-positive reaction in cement lines. PAS reaction, ×400.

Figure 10



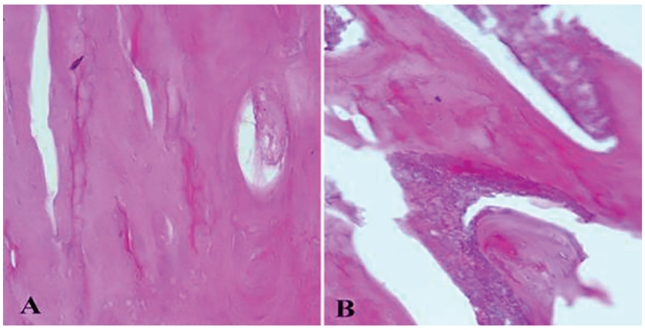
A longitudinal section of the middle shaft of the femur (a) and trabecular bone (b) of ovariectomized rats showing unapparent periodic acid Schiff (PAS)-stained section. PAS reaction, ×400.

Figure 11



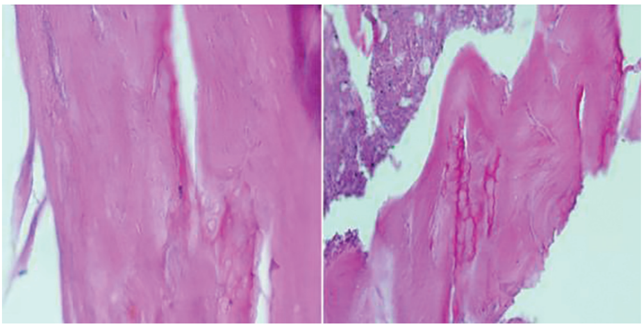
A longitudinal section of the middle shaft of the femur (a) and trabecular bone (b) of ovariectomized rats treated with synthetic estrogen compound showing very slight periodic acid Schiff (PAS)-positive reaction in cement lines. PAS reaction, ×400.

Figure 12



A longitudinal section of the middle shaft of the femur (a) and trabecular bone (b) of ovariectomized rats fed red clover exhibiting intense periodic acid Schiff (PAS)-positive reaction in cement lines (arrow). PAS reaction, ×400.

Figure 13



A longitudinal section of the middle shaft of the femur (a) and trabecular bone (b) of ovariectomized rats fed fennel exhibiting intense periodic acid Schiff (PAS)-positive reaction in cement lines. PAS reaction, ×400.

estrogen production is the predominant cause of rapid bone loss and deterioration of bone architecture, resulting in increased bone fragility during the first decade after menopause [35].

In the present study, ovariectomy of rats induced a decrease in thickness of the shaft cortical bone and decrease in the number of osteocytes. Many osteoporotic cavities were also seen. The results of the present work are in agreement with those of Weber *et al.* [36], Park *et al.* [37], and Kalleny [38], who reported that histomorphometric and statistical results of the outer cortical bone of OVX rats revealed significant decrease in the mean outer cortical bone thickness (28% loss) compared with sham-operated control rats.

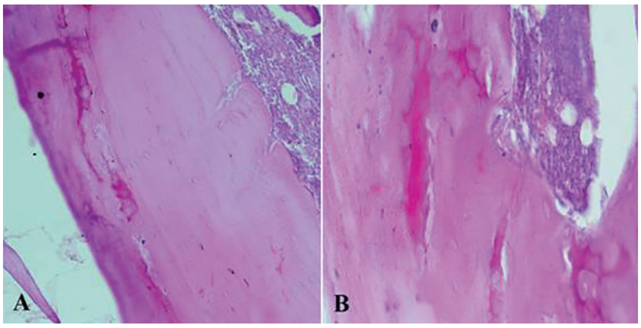
The cortical bone of OVX rats also showed resorption cavities and irregularly eroded endosteal surface containing osteoclasts [36] and reduction in the cortical and trabecular bone thickness [39].

Also, Khattab *et al.* [40] found that the femur cortical bone of OVX untreated rats showed osteoporotic regions with bone destruction, loss of normal Haversian system pattern, numerous resorption cavities, and distinct changes in femur cortical bone, as well as splitting and the presence of osteoclast cells.

Similarly NAMS [41] observed that estrogen deficiency triggers osteoclasts, which enhance bone loss by stimulating bone resorption.

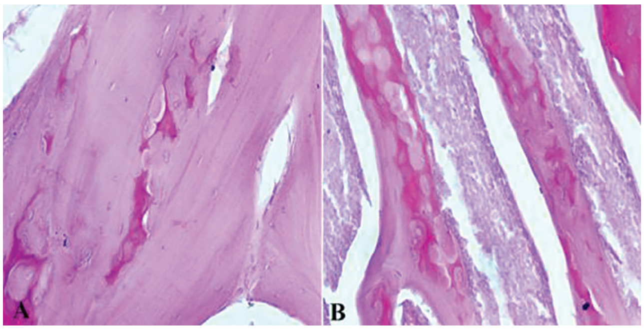
In the present study, the group of OVX rats showed marked decrease in the thickness of the bone trabeculae compared with sham-operated controls. The results of the present work are in agreement with those of Kalleny [38] who revealed that the group of OVX rats showed significant decrease in the mean trabecular bone volume (nearly 48% loss compared with controls) causing widening of the bone marrow spaces as a result of the increase in the intertrabecular distance. Moreover, these bone trabeculae showed loss of normal architecture in which some trabeculae were observed as islands of widely separated specules, whereas other trabeculae appeared thinned out.

Figure 14



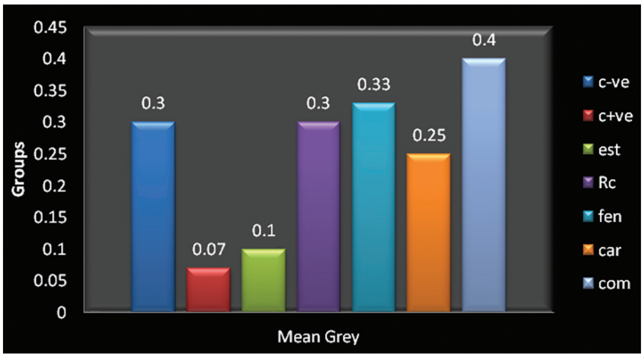
A longitudinal section of the middle shaft of the femur (a) and trabecular bone (b) of ovariectomized rats fed with carob exhibited moderate periodic acid reaction in cement lines positive reaction. PAS reaction, ×400.

Figure 15



A longitudinal section of the middle shaft of femur (a) and trabecular bone (b) of ovariectomized rats fed a combination of the three plants exhibiting very intense distinct periodic acid Schiff (PAS)-positive reaction in cement lines. PAS reaction, ×400.

Figure 16



A chart showing the mean gray level of periodic acid Schiff (PAS) stain in different groups.

The mechanism of ovariectomy-induced bone effect has been explained by Kimble *et al.* [42], who postulated that interleukin-1, a cytokine produced by bone marrow cells and bone cells, has a role in the pathogenesis of PMO because of its potent stimulatory effects on bone resorption *in vitro* and *in vivo*. Oktem *et al.* [43] have clarified that the pathogenesis of OP generates a free radical contributing to the imbalance between bone formation and resorption caused by estrogen depletion.

Reduction in estrogen leads to increased osteoclastic activity because of reduced hormonal control over osteoblast cell activity [44]. In the estrogen-deficient state, such as in menopause, the balance between bone resorption and bone formation shifts toward an increasing level of bone resorption, with more resorption than formation; this results in loss of bone mass and deterioration of trabecular bone microarchitecture [45].

In the present study, examination of bone sections of OVX rats treated with synthetic estrogen compound showed slight increase in the thickness of the cortical bone of the shaft, whereas the mean areas of Haversian canals were still reduced compared with that in OVX rats. Many osteoporotic cavities were still present. This may be explained by the findings of Li *et al.* [46], who reported that the reduction in the activation found with antiresorptive therapies with a transient increase in bone mass is due to the fill in of the resorption cavities.

The results of the present work are in disagreement with those of Hayashi *et al.* [47], who reported that histomorphometric indices of bone turnover were suppressed by treatment with estrogen.

Estrogen activation of osteoblasts stimulates expression of special proteins and growth factors [9]. Similarly, da Paz *et al.* [48] noted that estrogen had an independent anabolic effect on the osteoblastic function. Both ER activation and AR activation have the capacity to preserve trabecular bone mass [49].

Phytoestrogens are plant-derived nonestradiol phenolic compounds that are believed to protect against cardiovascular diseases, OP, and hormone-related disorders [50]. Phytoestrogens can be easily metabolized and eliminated. Many investigations have shown lower prevalence of OP and hip fracture among Asian women consuming high amounts of phytoestrogens [51].

Many types of phytoestrogens [52] are known to be antioxidant as they suppress the formation of ROS and prevent the release of cytochrome *c* from mitochondria. As previously known, OVX is associated with increase in free radicals [53]. These free radicals are responsible for causing physiological damage to many organs.

In the present study, the treatment of OVX rats with red clover resulted in an increase in cortical bone thickness as compared with the OVX group, and a noticeable increase in the number of osteocytes and decrease in the mean area of Haversian canals. No osteoporotic cavities or resorption cavities were recorded. The results of the present work are in agreement with those of Wronski and Yen [54], who reported that red clover is hypothesized to have a positive effect on BMD as women age. Similarly, Khattab *et al.* [40] found that the treatment of OVX with red clover resulted in protection from osteoporotic changes induced by OVX in a dose-dependent manner. The protective role of RCI may be attributed to its phytoestrogen effect on bone formation, and a consequence of a genomic and estrogen receptor-mediated effect [55]. In addition, Occhiuto *et al.* [5] found that treatment with isoflavones significantly reduced the number of osteoclasts compared with that in OVX control rats. These findings suggest that RCI are effective in reducing bone loss induced by ovariectomy, probably by reducing bone turnover through inhibition of bone resorption.

In addition, Sabudak and Guler [17] observed that red clover botanical dietary supplements have received a lot of attention recently for their potential use in the maintenance and improvement of bone health. It contains four important estrogenic isoflavones (daidzein, genistein, formononetin, and biochanin A) and coumestans. The red clover-induced improvement in bone histology has been explained by Polkowski and Mazurek [20], who postulated that the positive effect of isoflavones on bone metabolism may be mediated by at least two mechanisms: the first is the impact on osteoclasts through activation of apoptosis, and the second is the inhibition of tyrosine kinase activity through modulation of membrane ER with consecutive changes in the activity of alkaline phosphatase.

In the present study the treatment of OVX rats with fennel resulted in some improvement in histological changes in the form of increase in thickness of the shaft cortical bone and increase in the number of osteocytes, compared with OVX rats. The improvement in pathological changes in the present work may be due to the estrogenic potency of fennel. According to Oktay *et al*. [56], fennel seed extract has been shown to have estrogenic, antioxidant, and antihirsutism activities. Fennel extract is a rich source of phytochemicals, and many of these compounds have beneficial effects on human health. Jung *et al.* [57] demonstrated the role of estrogen as an antiosteoporotic agent. The effects of estrogen on osteoblasts and osteoclasts are mediated by binding to intracellular estrogen receptor and modulating the production of target proteins.

In addition, Djeridane *et al.* [58] reported that many of these phytochemicals possess significant antioxidant capacities that are associated with lower occurrence and lower mortality rates of several human diseases. The radical scavenging activity of the extracts could be related to the nature of phenolics and their hydrogen-donating ability. According to Yang *et al.* [59], scavenging of OH– is an important antioxidant activity of fennel because of its very high reactivity, which can easily cross the cell membranes at specific sites, react with most biomolecules, and cause tissue damage and cell death. Thus, removal of OH– is very important for the protection of the living system. According to Chatterjee *et al.* [60] the ability of fennel seed extracts to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the lipid peroxidation process, and to being good scavengers of active oxygen species, thus reducing the rate of chain reaction.

In the present experiment the treatment of OVX rats with carob induced an increase in shaft cortical thickness compared with OVX rats, and increase in the number of osteocytes denoting recovery of bone tissue. The mean areas of Haversian canals were still smaller in the OVX rats subjected to carob treatment as compared with untreated OVX rats. Increased ROS lead to oxidative stress and a degenerative signaling cascade triggered by oxidation of vital cellular components, which induced cellular damage and cell death [61]. Oxidative stress is characterized by depletion from intracellular stores of endogenous antioxidants or by rapid alteration in antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, resulting in increased lipoperoxidation [62]. The improvement in pathological changes observed in this group may be due to the antioxidant activity of carob. According to Rodrigo and Bosco [63] and Seifried *et al.* [64] the antioxidant capacity of carob extracts is mainly related to their higher level of phenolic compounds in this fraction. These compounds are well known for their ability to scavenge free radicals such as superoxide radical (O_2), hydroxyl radical (OH), and other ROS. Also, Rice-Evans *et al.* [65] reported that the ability of plant extracts to scavenge H_2O_2 may be attributed to their phenolic compounds, which donate electrons to H_2O_2 and reduce it to water. Sebai *et al.* [66] reported that *C. siliqua* L. pods (carob) contain antioxidants and vitamin E, and can help to improve bone fractures because the seeds are rich in phosphorus and calcium.

The richness of carob fruit [67] or leaf in polyphenols [24] is the basis of its antioxidant ability, scavenging free radicals such as hydroxyl radical (OH), which is the major cause of lipid peroxidation [68].

In the present study, the osteoporotic bone of OVX rats showed no signs of new bone deposition from the subperiosteal area, and the bone matrix did not show

PAS-positive reaction as compared with control. The results of the present work are in agreement with those of Kalleny [38], who reported that the bone of OVX rats showed no signs of subperiosteal bone deposition, and its matrix did not exhibit intense PAS-positive reaction compared with controls.

The compact and trabecular bones of OVX rats treated with red clover or fennel and/or carob exhibited intense PAS-positive reaction as compared with the bone of OVX rats. Chayanupatkul *et al.* [69] clarified that the newly formed bone takes on a distinctive magenta color from PAS reagent because the type of collagen matrix that is formed is type III, which is the emergency type, and a good candidate for repairing bone matrix. Type III collagen will then be replaced by the more permanent type I collagen matrix, which is the most stable because of its very strong cross-links allowing more stability for the new bone. In agreement with Ono *et al.* [70], who stated that collagen plays an important role in binding calcium in bone, it is suggested that the red clover, fennel, and carob treatment used in this study prevents the loss of BMD by upregulating collagen synthesis from osteoblasts of compact and trabecular bone. The area showing subperiosteal bone deposition as a distinct basophilic cement line also exhibited intense PAS-positive reaction.

Conclusion

Treatment of OVX rats with phytoestrogens such as red clover, fennel, and carob resulted in an improvement in the histopathological and histochemical changes and morphometric parameters in ovariectomy-induced OP. Thus, these phytoestrogens may contribute to the development of a new form of medicinal plant therapy in place of hormonal replacement therapy for menopause-induced OP.

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Conflicts of interest

There are no conflicts of interest.

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