

The Effect of *Ceratonia siliqua* Supplement on Bone Mineral Density in Ovariectomy-induced Osteoporosis in Rats

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Abstract. Aim: This study aimed to investigate the effect of *Ceratonia siliqua* on bone mineral density (BMD) as a non-pharmaceutical alternative treatment for postmenopausal osteoporosis. Materials and Methods: Thirty mature female Wistar rats were randomly separated into three groups of 10: Control, ovariectomized (OVX), and ovariectomized-plus-*C. siliqua* (OVX+CS). Total and proximal BMD were measured by dual-energy X-ray absorptiometry (DEXA) in all groups before ovariectomy, and at 3 and 6 months postoperatively.

At the end of the study, the femurs were subjected to a three-point bending test. Results: DEXA revealed no statistically significant difference in absolute values or percentage changes for total tibial BMD between OVX+CS and OVX groups throughout the study. In the proximal tibia, both absolute values and BMD percentage changes from baseline were higher in the OVX+CS group compared to the OVX group after 3 and 6 months of *C. siliqua* administration. Three-point bending test revealed a significantly higher thickness index in the OVX+CS group compared to the OVX group and a higher cross-sectional area index compared to the control group. Conclusion: Long-term administration of *C. siliqua* may be considered a non-pharmaceutical alternative treatment for postmenopausal osteoporosis. Further research is required to properly investigate the effects, and suitable treatment dose and schedule.

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Osteoporosis is a multifactorial skeletal disorder that is a public health concern and a heavy economic burden (1-3). After 35-40 years of age, osteoclasts are more active than osteoblasts. As a result, bone mass is lost, which occurs faster in postmenopausal women depending on lifestyle, diet, and other factors, because of their decreasing sex hormones

(4). In osteoporosis, the thickness and density of bone tissue are reduced and its microarchitectural structure is disrupted. This structural degeneration of bone tissue (deterioration of trabecular architecture) in combination with a low bone mass results in the bones becoming weaker, and porous, reducing their mechanical strength and flexural stress tolerance, increasing their fragility, as well as making them prone to fracturing (5). It is a 'silent' condition that does not warn of symptoms until a fracture occurs (6-9).

Early diagnosis of osteoporosis is particularly important, given the ever-increasing annual number of patients and the enormous consequences it has for their quality of life, society, and the economy (10). Because of the many consequences of osteoporosis, early and valid management is required. Osteoporosis management includes muscle strengthening, proper nutrition *i.e.*, adequate calcium, vitamin D and protein intake, and medications/drugs. Postmenopausal osteoporosis, where bone resorption exceeds bone formation, is mainly treated by drugs with antiresorptive properties. Estrogens, selective estrogen receptor modulators, bisphosphonates, strontium ranelate, denosumab, teriparatide, abaloparatide, or romosozumab are clinically used as successful therapies against postmenopausal osteoporosis. Nevertheless, their usage is linked to an established risk of side-effects after even a short period of administration. This, and patients' lack of compliance, make it imperative to expand the existing treatment options and develop new and alternative therapies. For these reasons, nutritional measures, either as alternatives or as supplements to pharmaceutical treatments, are being researched and proposed for postmenopausal women, such as fruits, vegetables, high protein diets, calcium, and vitamin D administration (4, 5, 11-13). Non-pharmaceutical products to support or provide alternate current treatments (5, 14) were the result of studying plant extracts, usually originating from folk medicine, with estrogenic, antioxidant and other beneficial properties regarding the prevention of diseases (2, 3, 15).

The use of phytoestrogens to replace hormone therapy is also being studied as an alternative treatment. Phytoestrogens imitating the action of estrogen hormones are thus considered natural selective estrogen receptor modifiers that are nevertheless weaker in terms of biological activity. Phytoestrogens such as isoflavones, lignans, and coumestans, have been found to affect bones by augmenting their strength and quality in a rat osteopenia model. Furthermore, phytoestrogens reduce the levels of bone turnover markers, augment bone mineral density (BMD), alleviate spinal bone loss in menopausal, and potentially protect from osteoporosis. Isoflavones restrain breast and prostate cancer. Flavonoids specifically have been found by many *in vitro* and *in vivo* studies to inhibit osteoclastogenesis and promote osteoblastogenesis (16-20). Up-regulation of estrogen

receptor β , resulting in augmented production of RUNT-related transcription factor 2/core-binding factor alpha 1 (21), activation of the signaling pathways that are mediated by cyclic adenosine monophosphate (22, 23), suppression of TNF superfamily member 11-mediated signaling pathways (24, 25), and enhanced intestinal calcium absorption (26), are considered possible mechanisms by which phytoestrogens achieve the aforementioned effects.

Despite continuous research on postmenopausal osteoporosis, its underlying pathogenic mechanism remains unclear. As a result, both its prevention and treatment are difficult to manage. Studies suggest that postmenopausal-induced endocrine derangement affects redox homeostasis, which is assumed to contribute, probably along with inflammation, to the development of associated diseases, such as postmenopausal osteoporosis. *Ceratonia siliqua* (carob) is a shrub with great amounts of antioxidant compounds, such as flavonoids and polyphenols, which have robust radical-scavenging activity (27-31). This is also implied by the protective effects of its extract and pods against oxidative stress in several organs (32-39). Thus, products from *C. siliqua* may be able to combat the damaging effects of free radicals, oxidative stress, and the reduced inflammation-induced response of antioxidants. In another study, a hydro-alcoholic extract from carob seeds increased levels of sex hormones (39). In Persian traditional medicine, carob fruit are used as an aphrodisiac and a treatment for male infertility (38). Aside from being beneficial for male fertility, traditional Turkish medicine considers it beneficial for health (38, 40, 41). It has also been correlated to the treatment of bleeding, and gastrointestinal, and kidney problems in Traditional Iranian Medicine (40, 42, 43). As well as its antioxidant and anti-diarrheal properties, a literature study by Karim and Azlan stated that *C. siliqua* also has anxiolytic/sedative, antidepressant, chemopreventive, and anticancer properties (44).

This study aimed to evaluate the osteoprotective effect of the *C. siliqua* beans on ovariectomized rats, which are the most widely used animal model for the study of postmenopausal osteoporosis (45-47). Ovariectomized animals are the most appropriate animal model available being representative of post-menopausal women. Such animal studies are pivotal for successful clinical trials and subsequent complication-free management of osteoporosis (16, 48-52). After the age of 10 months, bone remodeling is observed in female rats, which combined with post-ovariectomy bone loss, is ideal for the study of potentially osteoprotective substances. Twelve weeks post-ovariectomy, a decrease in trabecular bone volume is anticipated at the proximal tibia due to the rapid loss of trabecular microarchitecture caused by the declined ovarian production of estrogens. Therefore, the present study investigated the effect of *C. siliqua* on BMD in 10-month-old,



Figure 1. A brief outline of the experimental design. DEXA: Dual-energy X-ray absorptiometry.

ovariectomized female Wistar rats, as a non-pharmaceutical alternative treatment for postmenopausal osteoporosis.

Materials and Methods

Laboratory animals. In compliance with the European Directive 2010/63/EU, the present experimental protocol was approved by the General Directorate of Veterinary Services (permit no. 4505/10-7-2014). The PREPARE (53) and ARRIVE (54) guidelines were taken into consideration.

Thirty 3-month-old female Wistar rats with minimal body weight differences were purchased from the registered breeding unit of the Hellenic Pasteur Institute.

The animals were caged in groups of three or four, considering their body weight, in transparent polycarbonate open-top cages (dimensions 45×30×20 cm), under standard laboratory conditions (19-22°C, 55-65% relative humidity, 15 air changes per hour, 12-hour light/dark cycle). Standard maintenance rodent chow and tap water were supplied *ad libitum*. From the beginning to the end of the study, the rats were observed daily, with regular veterinary inspection and weekly body weight and food consumption measurements.

Study design. At the age of 10 months, the rats were allocated randomly into three groups of 10 rats each: Control, ovariectomy (OVX), and ovariectomy plus *C. Siliqua* (OVX+CS). Baseline body weight and BMD were measured. The control group provided information about the corresponding BMD changes in the same animal group (strain, origin, age, *etc.*), unaffected by ovariectomy or treatment. Bodyweight, food, and water consumption were measured every 2 weeks.

Two days postoperatively, all animals began to receive a diet free from soy and soy by-products. Food access of both OVX and OVX+CS groups was adjusted according to the control group's food intake to prevent obesity (55). Although pair feeding is not imperative, possible body weight deviations or post-ovariectomy obesity through *ad libitum* feeding can affect bone density and strength (56). Furthermore, pair feeding contributes to establishing the effect of *C. siliqua* on body weight or body composition free from alterations in energy intake (57).

All rats underwent three dual-energy X-ray absorptiometry (DEXA) measurements using a GE Lunar Prodigy Densitometer

(General Electric Healthcare, Madison, WI, USA) which includes special software for small animals, for assessing total and proximal tibial BMD. The first DEXA measurement was performed before ovariectomy for all animals at the age of 10 months; the next two measurements were conducted in the third and sixth month after the first measurement (resulting in timepoints of 0, 3, and 6 months). DEXA was performed under general anesthesia. After the third DEXA measurement, the animals were not allowed to recover but were subsequently euthanized with an additional dose (half of the initial dose) of anesthetic and blood was collected. The femurs were removed, dissected clean of tissues, and subjected to a three-point bending test. The experimental design is summarized in Figure 1.

One rat in the OVX group was euthanized according to our humane endpoints 6 days before the final DEXA measurement due to weight loss. Necropsy was not conclusive.

All procedures took place in the morning.

Rat chow preparation and administration. To avoid any potential bias, such as the potent estrogenic effect of the diet on BMD, all groups were provided with chow that was soy- and soy byproduct-free (4RF21; Mucedola S.r.l., Milan, Italy). The rat chow that was administered to the OVX+CS group was supplemented with *C. siliqua* (Locust beans, Haitoglou Family Foods, Thessaloniki, Greece). *Ceratonia siliqua* was mixed with food at a dose of 3 g/kg/day/rat, taking into consideration that a rat consumes about 20 g of food per day. The *C. siliqua* rat dose was calculated using the formula for the Human equivalent dose (HED): $HED (mg/kg) = \text{animal dose} (mg/kg) \times (\text{animal } K_m / \text{human } K_m)$, where the human K_m is 37 for a 60 kg human, animal K_m is G for rats; 0.5 g/kg/day was used as the HED (58-60). Its administration in food was preferred due to the composition of *C. siliqua* and the lack of the stress that other methods of administration are associated with; chronic physical stress is known to affects sex hormones. The rat chow was ground in a hammer mill through a 5-mm screen to become mash-like. This was mixed with powdered *C. siliqua* in a rotary mixer and pelleted using a Lister pellet press (Lister Co., Hardwicke, UK). *Ceratonia siliqua* was added to OVX+CS' food at a gradually increasing concentration on the third and fourth postoperative days for the animals to acclimatize to its taste.

Anesthesia for DEXA. The rats were anesthetized to remain immobile during the DEXA measurement. In the surgical preparation room, animals received intramuscular injections of

low doses of dexmedetomidine (Dexdomitor; Zoetis Hellas SA, Athens, Greece) and ketamine (Ketaset; Pfizer Hellas AE, Athens, Greece) (at 0.25 mg/kg and 50 mg, respectively). At the end of the measurements, the rats were transported back to the surgical preparation room, where anesthesia was reversed by intramuscular administration of atipamezole (Antisedan; Zoetis Hellas SA) at a dose of 1 mg/kg. The animals were then kept under close supervision until they were fully recovered. After the clinical confirmation of their recovery, they were transferred back to their cages.

Throughout each anesthesia, the rats' eyes were protected by regular application of lubricant eye ointment, as their eyes remained open during anesthesia, which could cause dryness and injury to the cornea.

DEXA. After anesthesia was established, the rats were transferred to the DEXA machine bed. In advance of each group's measurement, the system was calibrated (45).

DEXA software estimates the bone mineral content in a region of interest (ROI) and divides it by the area of the ROI (cm²) to determine the BMD (61, 62). In the current study, two ROIs were determined using the software's corresponding ROI tool. The entire tibia consisted of the first ROI (ROI1), including both cortical and trabecular bone. A region near (3 mm) the tibial plateau comprised the second ROI (ROI2: 0.19×0.19 mm²) to represent the proximal tibial metaphysis that is rich in trabecular bone. The same, blinded operator performed the selection of all ROIs at the end of the DEXA study. The *in vitro* precision (coefficient of variation) of the system was 0.5%.

Ovariectomy. Ovariectomy was performed under aseptic procedures, 10 days after the first DEXA measurement, so that the rats had fully recovered. The control group was sham-operated. The animals underwent the same anesthesia as for the DEXA measurements. In addition, rats were subcutaneously administered preoperative analgesia by carprofen (Rimadyl; Zoetis Hellas SA), and chemoprophylaxis by enrofloxacin (Baytril; Bayer, Leverkusen, Germany) at doses of 4 mg/kg and 10 mg/kg, respectively.

The rats were placed on a warming pad on the surgical table to prevent hypothermia due to anesthesia. An incision was made at the prepared surgical site (clipped, scrubbed/disinfected, and draped) midway between the umbilicus and pubis. Once the subcutaneous connective tissue was cut open, another incision was made in the *linea alba*. After entering the peritoneal cavity, the left ovary was located, the ovarian fat pad surrounding the ovaries was retracted, the broad ligament was separated, and two ligations were placed in the corresponding ovarian and suspensory ligaments, with an absorbable 4-0 suture. The ovary was then removed. The same procedure was repeated for the right ovary. The surgical wound was then closed in layers, with simple interrupted sutures, using an absorbable 4-0 suture. On the skin though, non-absorbable sutures were used.

Ovariectomy was confirmed at the end of the study, at necropsy, by the presence of uterine atrophy and the absence of ovarian tissue.

Euthanasia and tissue sampling. Following general anesthesia at the final DEXA measurement, the animals were euthanized and blood was collected *via* the posterior *vena cava*. Blood was collected in EDTA-coated tubes and centrifuged. The plasma was stored at -80°C in Eppendorf tubes. Necropsy followed, to assess the OVX surgery and check for potential pathological findings.

The following organs were collected, examined, and weighed when euthanasia was confirmed, in a single-blind procedure: Liver, brain, bladder, heart, right kidney, uterus, small intestine, and breast. Organs were weighed because a change in the weight of an internal organ can be the only sign of pathology. Special care was given to handling the tissues quickly since once removed from the body, they desiccate and lose weight, which is especially important for small organs like the uterus (63, 64). Furthermore, to determine whether *C. siliqua* had a direct effect on an organ, the ratio of the organ weight to the animal's final body weight, known as the relative organ weight, was calculated and analyzed.

The tibiae were extracted and after quickly removing the surrounding tissues, the right tibia was placed in an alcohol vial and the left tibia in formalin solution. The dissected femurs were also folded with gauze, soaked in isotonic saline (0.9%) to retain their moisture, placed in special containers and preserved at -20°C. The femurs were sent to the Unit of Biomechanics, Department of Mechanics, School of Applied Mathematical and Physical Sciences, National Technical University of Athens, to assess their mechanical strength through a three-point bending test.

The plasma and the organs remain available for further research, reducing the number of animals used in research.

Three-point-bending test. *Ex vivo*, a three-point bending test was used to evaluate the bone's mechanical properties. This test determines the bending strength of the bone or in other words the maximum load that can be sustained by the bone before its fracture. Furthermore, this test was selected because it is easy to apply to rats, the distribution of the internal loading is similar to the physiological one, and it produces robust measurements with low variation of the callus shape.

On the day of the test, the packaged femurs were left at ambient temperature to thaw, which does not significantly affect the bone's mechanical properties. As before with DEXA, the equipment was calibrated ahead of every test.

The three-point bending test was carried out using a Materials Test System (Eden Prairie, MN, USA) electromechanical frame. The bones were placed on two cylindrical supports (their distance was equal to 16 mm) while a rounded punch was used to apply the load at the bone's mid-span. A displacement-control loading scheme was adopted. The loading rate was constant throughout all the experiments, equal to 0.1 mm/min, definitely ensuring quasi-static loading conditions.

Before each test, the line of action of the force applied concerning the orientation of the loaded cross section was marked. During the tests, the deflection at the central cross-section of the bones (*i.e.*, the section at which the load was applied) was recorded with the aid of a properly calibrated video-extensometer (RTSS video-extensometer; Limes Messtechnik & Software GmbH, Krefeld, Germany). In addition, the load applied was also measured (with the aid of a calibrated load cell) and recorded as a function of time, continuously up to the fracture of the specimens.

After each test, one of the two bone fragments was used for further biomechanical study. The fragment was vertically placed in a plastic cup which was then filled with molten resin. After curing the resin, the construct (bone fragment and resin) was removed from the cup and its free surface was carefully polished using increasingly fine abrasive papers. The polished surface was then photographed using a stereoscope.

Biomechanical analysis. The photographs from the stereoscope were processed using commercially available software. As a first step, the geometry of the cross-section of fractured bone (*i.e.*, outer and inner perimeters) and the loading axis, were drawn. Proper elaboration of these drawings provided the: (i) Cross-sectional area, (ii) coordinates of the centroid of the cross-section with respect to an auxiliary reference system, (iii) eccentricity of the loading axis with respect to the centroid, (iv) average thickness of the cortical bone, and (v) area enclosed by the median line.

As a second step, a centroidal reference system $x_c y_c$ was introduced, with its y_c -axis parallel to the loading line, as it is the standardized procedure for the analysis of bending tests according to the classical Bernoulli-Euler technical theory (65). Then, the tensor of the second moments of area (namely, the quantities $I_{x_c x_c}$, $I_{y_c y_c}$, $I_{x_c y_c}$) and the corresponding principal second moments of area (*i.e.*, $I_{x_p x_p}=I_{min}$, $I_{y_p y_p}=I_{max}$) were determined, together with the orientation of the respective axes.

The bending moments, M_x and M_y , about the principal axes were then calculated by means of equations:

$$M_y = \frac{P_x L}{4}, M_x = \frac{P_y L}{4} \quad (\text{Eq. 1})$$

where P_x and P_y are the Cartesian components along the two principal axes of the force P applied and L is the distance between the two supports.

In addition, the respective (parasitic) torsional moment was calculated as:

$$M_{\text{torsional}} = Pe \quad (\text{Eq. 2})$$

where e is the eccentricity of the load line with respect to the centroid of the cross-section.

Assuming the linear response of the bones (an assumption quite reasonable for a brittle material like bone tissue) and adopting the technical bending theory introduced by Bernoulli and Euler (65), the equation of the neutral line (*i.e.* the line on which the axial normal stress is zero) was determined as:

$$\sigma_{\text{bending}} = 0 \Rightarrow \pm \frac{M_x}{I_{x_p x_p}} y \mp \frac{M_y}{I_{y_p y_p}} x = 0 \quad (\text{Eq. 3})$$

where the “+” and “-” signs are interchanged, depending on whether the respective stress component is of tensile or compressive nature. Finally, the maximum normal (tensile) stress, namely the stress developed at the critical point of the section (*i.e.*, the point most distanced from the neutral line) was calculated as follows:

$$\sigma_{\text{bending}} = \pm \frac{M_x}{I_{x_p x_p}} y_k \mp \frac{M_y}{I_{y_p y_p}} x_k \quad (\text{Eq. 4})$$

where x_k and y_k correspond to the coordinates of the critical point. The respective (parasitic) shear stress was calculated as:

$$\tau_{\text{torsional}} = \frac{M_{\text{torsional}}}{2A_m t} \quad (\text{Eq. 5})$$

where A_m is the area encircled within the mean line of the specimen’s cross-section (65).

It is recalled here that the generation of parasitic shear stresses given by Eq. 5 is caused by the inevitable eccentricity between the loading axis and the centroid of the cross-section, and (as will be seen in the next sections) their magnitude is by no means negligible. On the contrary, the shear stresses caused by the shear force itself are ignored, given that the restrictions of the technical bending theory concerning the length-over-height ratio, are definitely fulfilled by all the specimens of the present experimental protocol.

Statistical analysis. The number of rats required for the study was determined by sample size estimation using G*Power 3.1.9.2 program (G*Power - Universität Düsseldorf: Psychologie - HHU, Düsseldorf, Germany) (66). A sample size of 10 animals per group offers an 80% probability of showing a statistically significant difference of 15% between groups (OVX: -35% versus OVX+CS: -20%, standard deviation 10%), in the percentage change from baseline to 6 months for proximal tibial BMD with a significance level of 1.7% (Bonferroni correction for three groups). The individual rat was used as the experimental unit.

Data are expressed as mean±standard deviation. Shapiro-Wilks test confirmed the parameters’ normal distribution.

We used the two-way mixed analysis of variance (ANOVA) model using the intervention (between groups) and time (within a group) as factors for the analysis of BMD measurements using the Bonferroni correction for all pairwise comparisons whether between or within groups.

The comparison of three-point bending results for each femur, body weight, the organs weight, and the relative organ weight between groups were performed using the one-way ANOVA model. Pairwise comparisons were performed using Bonferroni test.

Sensitivity analysis of BMD measurements, concerning baseline balance between groups, was performed using two methods:

i) The mean percentage change from baseline after 3 and 6 months respectively where comparison of percentage change from baseline of BMD parameters during the observation period between groups was analyzed using the one-way ANOVA model, and pairwise comparisons were performed using the Bonferroni test. Kruskal-Wallis and Mann-Whitney tests were used in the case of violation of normality.

ii) The absolute change from baseline after 3 and 6 months using analysis of covariance model using the absolute change from baseline as the dependent variable, the groups as a factor, and the baseline value of the measures as covariate.

All tests were two-sided, and statistical significance was set at $p < 0.05$. All analyses were carried out using the statistical package SPSS VR 21.00 (IBM Corporation, Somers, NY, USA).

Results

DEXA measurements. The DEXA results for total tibial BMD are presented in Table I. Throughout the study, there was a statistically significant interaction between the factors intervention and time ($p < 0.005$) for total and proximal tibial BMD.

Total tibial BMD. Differences within groups: In the control group, there was no significant difference between measurements over time. In the OVX group, the value

Table I. Comparison of tibial bone mineral density (BMD, g/cm²) between control, ovariectomized (OVX) and ovariectomized plus *Ceratonia siliqua*-treated (OVX+CS) groups during the study period.

Tibial BMD	Group	Baseline	3 Months	6 Months
Total	Control	0.228±0.011	0.235±0.012	0.237±0.011
	OVX	0.236±0.021	0.217±0.010 ^{a,c}	0.204±0.012 ^{a,c,d}
	OVX+CS	0.232±0.018	0.215±0.010 ^{a,c}	0.204±0.014 ^{a,c,d}
Proximal	Control	0.363±0.043	0.380±0.030	0.382±0.033
	OVX	0.374±0.048	0.255±0.033 ^{a,c}	0.243±0.027 ^{a,c}
	OVX+CS	0.355±0.034	0.300±0.018 ^{a,b,c}	0.283±0.022 ^{a,b,c,d}

Significantly different at $p < 0.05$ versus: ^aControl, ^bOVX, ^cbaseline, and ^d3 months. All values are presented as the mean±standard deviation.

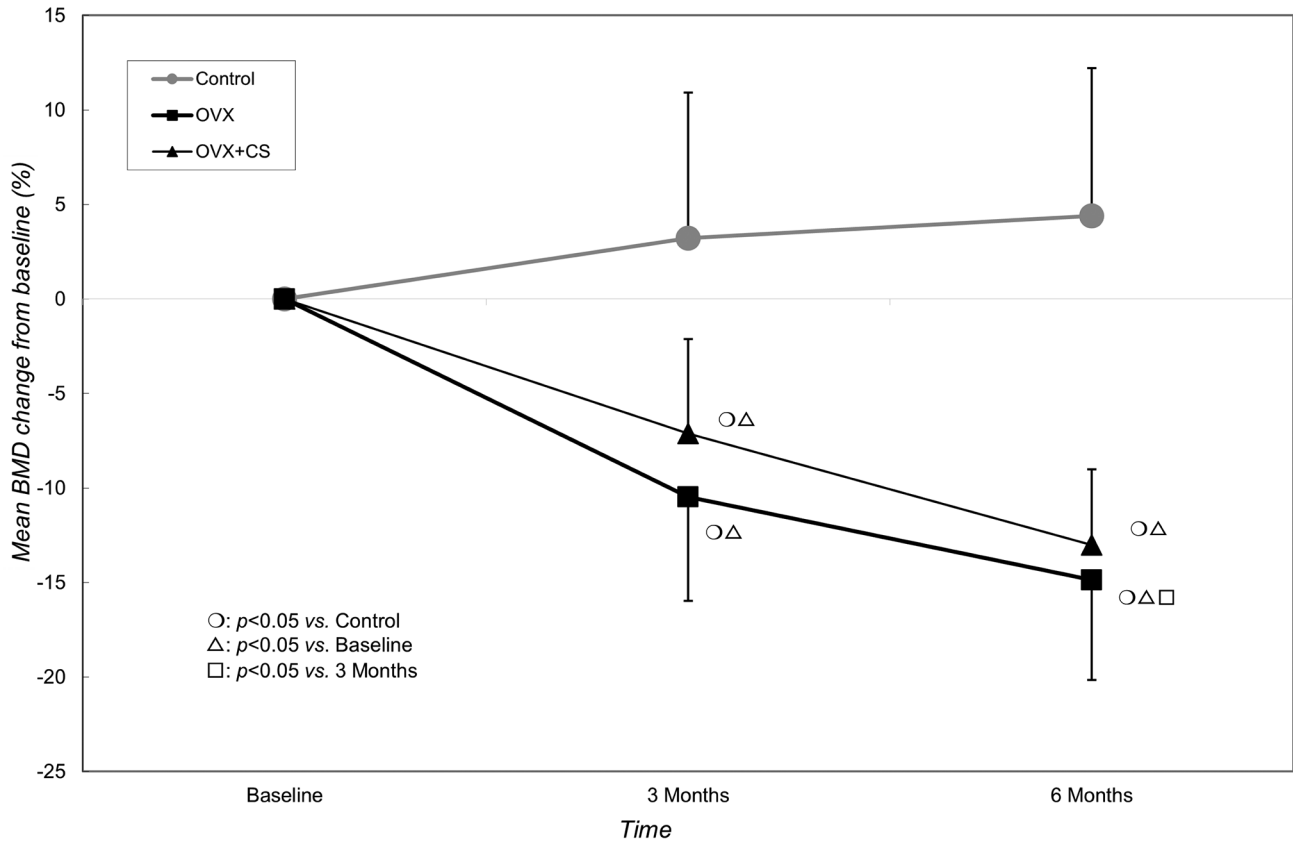


Figure 2. Comparison of mean percentage change from baseline in total tibial bone mineral density (BMD) in control, ovariectomized (OVX) and ovariectomized plus *Ceratonia siliqua*-treated (OVX+CS) groups during the study period.

decreased significantly in both the third ($p < 0.005$) and sixth ($p < 0.005$) month. The decrease between third and sixth month was also significant ($p < 0.005$). The total tibial BMD value of the OVX+CS group at baseline was significantly higher compared to those at both 3 ($p < 0.005$) and 6 ($p < 0.005$) months. Additionally, the total tibial BMD of the OVX+CS group at 3 months was significantly higher than that at 6 months ($p < 0.005$).

Differences among groups: At baseline, there was no statistically significant difference between the groups. The control group presented statistically significant higher values compared to the other two groups at both the third ($p < 0.005$) and sixth ($p < 0.005$) month, whilst there were no significant differences noted between the OVX and OVX+CS groups throughout the study period ($p > 0.99$). Independently of time, the control group presented higher BMD values

Table II. Comparison of absolute differences from baseline at 3 and 6 months in total and proximal tibial bone mineral density (BMD, g/cm²) between control, ovariectomized (OVX) and ovariectomized plus *Ceratonia siliqua*-treated (OVX+CS) groups during the study period.

Tibial BMD	Group	Mean absolute difference from baseline (95% CI)	
		At 3 months	At 6 months
Total	Control	0.004 (-0.001-0.010)	0.007 (0.001-0.013)
	OVX	-0.016 (-0.022-0.011) ^a	-0.031 (-0.036-0.025) ^a
	OVX+CS	-0.017 (-0.022-0.011) ^a	-0.017 (-0.034-0.023) ^a
Proximal	Control	0.016 (-0.002-0.034)	0.019 (0.003-0.034)
	OVX	-0.111 (-0.129-0.093) ^a	-0.125 (-0.140-0.109) ^a
	OVX+CS	-0.063 (-0.081-0.045) ^{a,b}	-0.078 (-0.093-0.062) ^{a,b}

CI: Confidence interval. Significantly different at $p < 0.05$ versus: ^aControl, and ^bOVX.

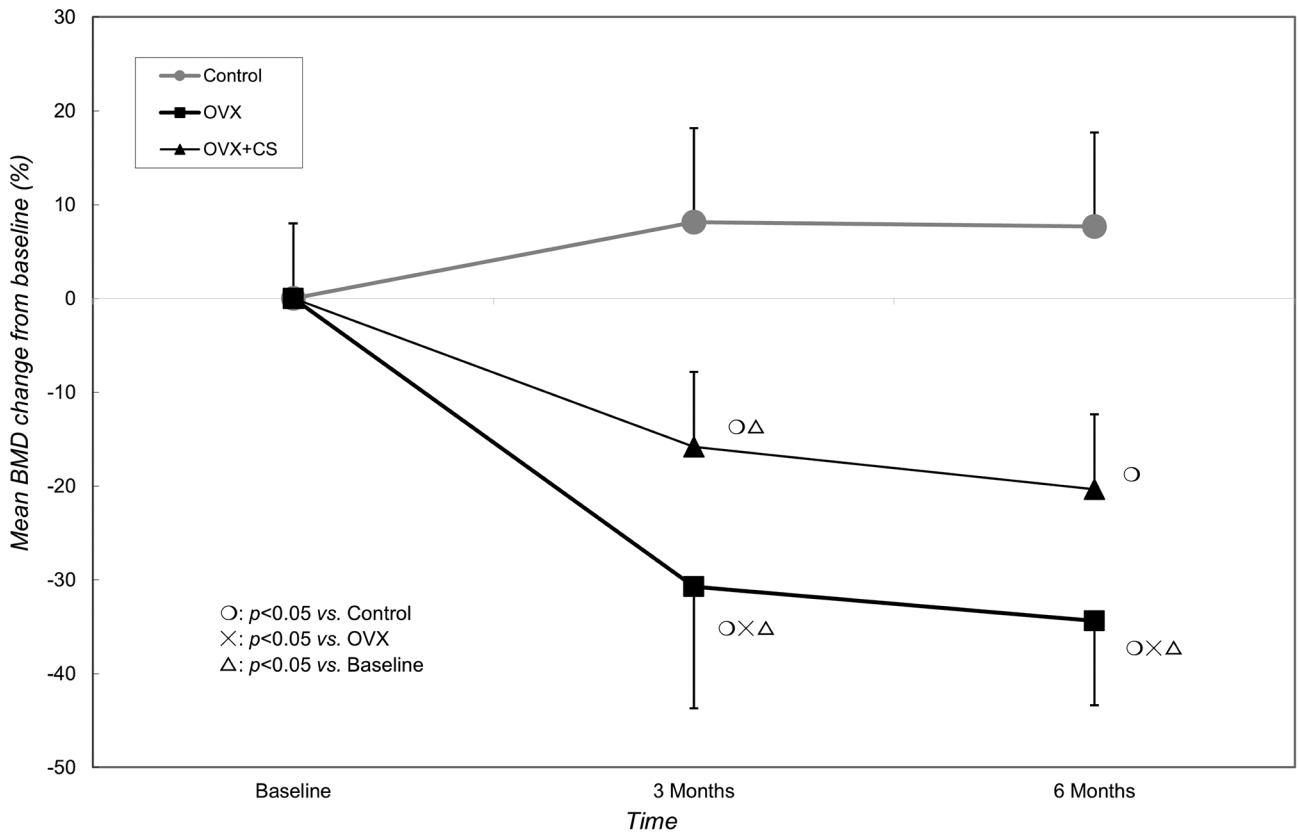


Figure 3. Comparison of mean percentage change from baseline in proximal tibial bone mineral density (BMD) in control, ovariectomized (OVX) and ovariectomized plus *Ceratonia siliqua*-treated (OVX+CS) groups during the study period.

(0.233±0.004) compared to both OVX (0.219±0.004, $p=0.040$) and OVX+CS (0.217±0.004, $p=0.016$) groups, while there were no significant differences noted between the last two groups ($p > 0.99$).

Change in total tibial BMD from baseline. The control group displayed a significantly increased BMD compared to the

other groups both at 3 ($p < 0.005$) and at 6 ($p < 0.005$) months. The change in BMD for the OVX+CS group did not differ significantly from that of the OVX group at 3 months (-6.84±4.96 vs. -7.62±4.97, $p > 0.99$) nor at 6 months (-11.98±2.75 vs. -13.53±4.94, $p > 0.99$) (Figure 2).

The absolute differences in total tibial BMD from baseline during the observation period (Table II) followed a similar

Table III. Results of the three-point-bending test in control, ovariectomized (OVX) and ovariectomized plus Ceratonia siliqua-treated (OVX+CS) groups.

Femur	Index	Group			p-Value
		Control	OVX	OVX+CS	
Right	von Mises stress, MPa	191.92±35.03	179.90±34.75	199.97±53.07	0.588
	Bending stress, MPa	191.81±34.97	179.68±34.78	199.66±53.00	0.589
	Torsional stress, MPa	3.13±2.37	3.95±3.26	4.79±4.67	0.589
	Cross-sectional area, mm ²	5.02±0.45	5.07±0.23	5.14±0.50	0.093
	Thickness, mm	0.67±0.09b	0.58±0.05	0.64±0.04b	0.010
Left	von Mises stress, MPa	223.67±44.90	198.18±22.87	202.14±40.18	0.292
	Bending stress, MPa	223.50±44.93	197.80±22.99	201.81±39.96	0.285
	Torsional stress, MPa	4.26±2.75	5.75±4.19	5.58±4.57	0.658
	Cross-sectional area, mm ²	4.81±0.40	5.17±0.36	5.29±0.46a	0.037
	Thickness, mm	0.63±0.09	0.59±0.07	0.67±0.07	0.101

Significantly different at $p < 0.05$ versus: ^aControl, and ^bOVX. All values are presented as the mean±standard deviation.

pattern to that shown in the analysis of the mean percentage change in total tibial BMD.

Proximal tibial BMD. Differences within groups: In the control group, there were no significant differences noted for the proximal tibial BMD during the observation period. The baseline value of the OVX group was significantly higher compared to that at both 3 ($p < 0.005$) and 6 ($p < 0.005$) months. Within the OVX+CS group, the value of the proximal tibial BMD was significantly higher at baseline compared to those at 3 and 6 months (both $p < 0.005$). For both OVX ($p = 0.235$) and OVX+CS ($p = 0.076$) groups, there were no significant differences noted between the BMD values in the third and sixth month.

Differences among groups: At baseline, there was no statistically significant difference between the groups. The control group had a significantly higher value compared to OVX and OVX+CS at 3 and 6 months (both $p < 0.005$). Likewise, the proximal tibial BMD value of the OVX+CS group was higher than OVX's group at 3 months ($p < 0.005$) and at 6 months ($p = 0.009$). Regardless of time, the control group (0.375 ± 0.008) presented significantly higher BMD values compared to both OVX (0.291 ± 0.008 , $p < 0.005$) and OVX+CS (0.312 ± 0.008 , $p < 0.005$) groups, and there were no significant differences noted between the last two groups ($p = 0.225$).

Change in proximal tibial BMD from baseline. The proximal tibial BMD of the control group was significantly higher compared to the other groups at 3 and 6 months (both $p < 0.005$) (Figure 3). The BMD for the OVX+CS group was statistically significantly higher compared to the OVX group at both 3 months (-15.14 ± 7.56 vs. -30.72 ± 13.64 , $p = 0.009$) and 6 months (-19.79 ± 7.42 vs. -34.37 ± 9.44 , $p < 0.005$).

Table IV. Comparison of bodyweight results (g) in control, ovariectomized (OVX) and ovariectomized plus Ceratonia siliqua-treated (OVX+CS) groups.

Week	Group			p-Value
	Control	OVX	OVX+CS	
0	286.4±28.8	297.7±30.2	292.5±21.4	0.656
4	293.6±23.6	315.0±34.7	300.3±33.2	0.108
6	304.0±28.6	335.4±39.7	320.0±28.9	0.112
8	310.5±31.3	351.60±45.0	333.5±31.9	0.062
10	317.0±36.3	358.4±45.3	341.9±31.8	0.070
12	319.0±29.9	366.3±48.3a	349.7±34.1	0.032
14	323.3±24.2	374.2±51.5a	357.5±36.6	0.022
16	321.4±31.9	377.6±55.1a	360.5±35.8	0.018
20	323.7±34.3	395.3±56.7a	369.9±39.2	0.004
24	352.9±28.5	413.0±59.1a	379.3±43.1	0.022

^aSignificantly different at $p < 0.05$ versus Control. All values are presented as the mean±standard deviation.

The absolute differences in proximal tibial BMD from baseline during the observation period (Table II) followed a similar pattern to that shown in the analysis of the mean percentage change in proximal tibial BMD.

Three-point-bending test. The between-group comparison did not show a difference for von Mises stress, bending stress, torsional stress, and cross-sectional area. The test results are displayed in Table III. The thickness index was significantly higher in the OVX+CS ($p = 0.026$) and control ($p = 0.029$) groups compared to the OVX group for the right femur. Regarding the left femur, the cross-sectional area index ($p = 0.037$) was marginally significantly higher in the OVX+CS group ($p = 0.041$) than that of the control group.

Table V. Comparison of organ weights (g) in control, ovariectomized (OVX) and ovariectomized plus *Ceratonia siliqua*-treated (OVX+CS) groups.

Tissue	Group			p-Value
	Control	OVX	OVX+CS	
Heart	0.833±0.134	0.936±0.098	0.880±0.104 ^b	0.163
Left kidney	1.279±0.153	1.057±0.080 ^a	0.953±0.116 ^a	<0.05
Abdominal fat	23.16±3.35	38.91±11.23 ^a	27.55±7.48 ^b	<0.05
Gastrocnemius muscle	1.786±0.143	1.676±0.325	1.714±0.125	0.518
Brain	1.947±0.068	2.072±0.087 ^a	1.923±0.144 ^b	0.011
Uterus	0.573±0.107	0.197±0.054 ^a	0.133±0.028 ^a	<0.05
Liver	10.91±1.47	9.17±1.23 ^a	8.91±1.58 ^a	0.010

Significantly different at $p<0.05$ versus: ^aControl, and ^bOVX. All values are presented as the mean±standard deviation.

Body, organ, and relative weights.

Bodyweight. From baseline until the 10th week, there was no statistically significant difference in body weights between groups. In contrast, from the 12th week until the end of the study, there was a statistically significant difference in body weights between the groups: the rats in the control group presented statistically significantly lower body weight compared to those of the OVX group ($p<0.05$). From the 12th week onwards, animals in the OVX+CS group had similar body weights to animals of the control and OVX groups. Mean body weight changes over the duration of the study are displayed in Table IV.

Organ and abdominal fat weight. Uterine, liver, and kidney weights did not differ between OVX and OVX+CS groups. The significantly lower uterine weight of the OVX ($p<0.005$) and OVX+CS ($p<0.005$) groups in comparison to that of the control group confirmed that OVX was successful (Table V). The brain weighed significantly less in the control ($p=0.046$) and OVX+CS ($p=0.014$) groups compared to the OVX group, whilst the abdominal fat had a similar weight in the control and OVX+CS groups.

Relative weights. The relative abdominal fat weight was significantly higher in the OVX group compared to the control ($p=0.001$) and OVX+CS ($p=0.009$) groups, which were similar (Table VI). No difference was noted in the relative weights of the uterus, liver, and kidneys between OVX and OVX+CS groups either, meaning that *C. siliqua* does not appear to affect them. Relative heart and brain weights were also similar among all groups. The OVX+CS group had a lower relative organ weight compared to the control group regarding the kidneys

Table VI. Comparison of relative organ weight (organ weight/body weight) between control, ovariectomized (OVX) and ovariectomized plus *Ceratonia siliqua*-treated (OVX+CS) groups.

Tissue	Group			p-Value
	Control	OVX	OVX+CS	
Heart	0.235±0.024	0.226±0.025	0.233±0.025	0.678
Left kidney	0.362±0.030	0.256±0.03 ^a	0.252±0.026 ^a	<0.05
Abdominal fat	6.54±0.65	9.10±1.66 ^a	7.18±1.34 ^b	<0.05
Gastrocnemius muscle	0.507±0.033	0.396±0.073 ^a	0.455±0.041	<0.05
Brain	0.556±0.059	0.504±0.086	0.511±0.057	0.211
Uterus	0.165±0.041	0.048±0.012 ^a	0.035±0.005 ^a	<0.05
Liver	3.086±0.271	2.205±0.265 ^a	2.346±0.281 ^a	<0.05

Significantly different at $p<0.05$ versus: ^aControl, and ^bOVX. All values are presented as the mean±standard deviation.

($p<0.005$), uterus ($p<0.005$), and liver ($p<0.005$). The OVX group had lower relative weight compared to the control group regarding the kidneys ($p<0.005$), uterus ($p<0.005$), liver ($p<0.005$), and gastrocnemius ($p<0.005$).

Discussion

The role of estrogens in regulating menopause symptoms is supported by increasing data (67, 68). In menopause, estrogen levels decrease, bone resorption exceeds bone formation, BMD decreases, and, subsequently, the risk of fracture increases. Most current therapies exert mainly anti-resorptive effects (68-71). However, estrogen administration is associated with major side-effects such as stroke, thromboembolism, breast cancer, and vascular diseases (17, 68, 72). Therefore, plant-extracted non-steroidal, bioactive substances that resemble, or modulate endogenous estrogens and are devoid of the aforementioned hazards, are considered a safe and effective complementary medicine for managing postmenopausal symptoms (73, 74). Women's preference for phytoestrogens along with their health benefits have led to increased attention to these compounds (67, 75).

Isoflavone aglycones have already been found to have a positive effect on estrogen deficiency-induced bone loss (76). *Ceratonia siliqua* is rich in flavonoids (free and glycosylated aglycone). Vaya and Mahmood identified nine different flavonoids in its leaves (77). Hence, here *C. siliqua* was studied for a 6-month period for its potential osteoprotective effect against postmenopausal BMD loss in an ovariectomized rat model, commonly used for that purpose in research on postmenopausal osteoporosis.

The impact of ovariectomy on bone mineral and microarchitecture differs among skeletal sites (78) and maybe this is the reason for some diverse reactions of sites to osteoporosis management (79). The proximal tibia of the rat is the first to display differences after ovariectomy, as well as the greatest changes, compared to the spine, distal femur, and proximal femur. The proximal tibia also presents the highest BMD values before ovariectomy, and the greatest BMD loss after. Specifically, the microarchitecture of the metaphysis of the proximal tibia deteriorates. Rat tibial metaphyseal trabeculae are more prone to estrogen deficiency-induced bone loss (not its thickness) and strength changes by trabecular hypertrophy or hyper-mineralization (78, 80). Trabecular bone is highly correlated with mechanical performance and bone strength. In particular, trabecular number and connectivity density [number of trabecular connections per cubic millimeter (81)] are more closely related to BMD than trabecular thickness is. In contrast, epiphyseal trabeculae are primarily strengthened by thickening and their greater BMD is unaffected by time. Such variations may result from different mechanical loads (80, 82, 83). The reaction of each trabecular bone region also depends on age. In a study by Francisco *et al.*, 24- and 44-week-old rats presented a more reliable osteoporotic reaction to ovariectomy (reduced bone mineral and microarchitectural properties) than younger ones (reduced trabecular connectivity and trabecular morphological change from plate-like to rod-like) (78, 84-88).

Similarly to the findings of Francisco *et al.*, significant amelioration was found for proximal tibial BMD loss in our study whilst there were no significant differences between OVX and OVX+CS groups regarding total tibial BMD. Both in the third and sixth month, *C. siliqua* administration better maintained proximal tibial BMD, whose absolute value was higher in the OVX+CS group especially in the third month, in comparison to the OVX group. Protection of proximal tibial BMD after *C. siliqua* administration was also confirmed by its percentage changes that showed augmentation compared to the OVX group in the third month, which became significantly higher in the sixth month of administration. Thus, this inhibition of the loss of trabecular bone volume in the proximal tibia may represent a potentially protective effect of *C. siliqua* against postmenopausal osteoporosis.

Bone strength is determined by its quantitative (bone mass, bone density, and modulus of elasticity) and qualitative properties such as trabecular bone structure features (trabecular orientation and thickness, length, number, and separation distances of trabeculae). Specifically, in the three-point bending test, the cortical mineral content at the middle of the diaphysis, the cortical bone cross-section surface, the percentage cortical bone cross-section surface to whole cross-section surface, the cortical density, and the thickness

of the cortical bone, are considered to be markers of bone mechanical strength (89-91). Cortical and trabecular thinning are noted in studies of both ovariectomized animals and postmenopausal women (92-95). According to Jiang *et al.*, ovariectomy in rats causes a considerable decrease in the total BMD but does not affect the cortical bone thickness, cortical BMD, or cortical bone area (96). Nevertheless, total BMD, cortical bone area, and cortical bone thickness were significantly correlated to femoral failure load, implying that they can be used as its indicators. Cortical bone thickness exhibited the greatest association with failure load in the same study. It has also been reported that dimple fracture is affected by bone thickness (97). This highlights the significance of the three-point bending test result that administration of *C. siliqua* significantly increased the thickness index compared to the OVX group for the right femur, even though it remained significantly lower compared to the control group when the index was examined regardless of the foot factor. Moreover, femurs are considered the optimal rodent bone for evaluating fracture toughness properties in small animal model studies (98).

The bone cross-sectional area is an indicator of axial strength, and cortical bone mass, and greatly influences the bone strength determination. The cross-sectional cortical area at the midshaft is considered the best predictor for the elastic modulus of the femur (99, 100). Thus, the significantly lowered cross-sectional area index of the control group compared to the OVX+CS group for the left femur may be another indication of the potent osteoprotective effect of *C. siliqua*. This result becomes more significant considering that the three-point bending test assesses mechanical properties of femoral cortical bone since it consists of the greatest part of the femoral midshaft (101-103). In rats, cortical bone responds slowly to ovariectomy in comparison to trabecular bone, especially in rats more than 9 months old (104). Consequently, longer (>6 months) study periods and a more sensitive detection approach in the case of older animals may be the reasons that no other statistically significant changes regarding biomechanical testing were observed among groups in such osteoporotic studies (105-107). The limited duration of study as a possible cause of the three-point bending results has been well-discussed by Zervas *et al.* (102). However, a transient rise in cortical bone strength of the femoral diaphysis that declines after 9 months has been reported (45). Likewise, an increase in cortical thickness with aging has been reported and associated with the "cortical drift" phenomenon (108, 109). It has also been reported that the cross-sectional area index increases as animals age (100, 107) and that it is affected by the bodyweight of animals (101).

All animals displayed similar body weights from the 12th week on; animals in the control group weighed significantly less than the animals in the OVX group but there was no

significant difference when comparing the weights of the animals of the OVX+CS group to those of the control and OVX groups. Bodyweight is mainly affected by growth factors. Consequently, it is not correlated to uterine weight, which is influenced by hormones such as estrogens (64). The uterus reacts to estrogens by increasing its weight, initially due to water imbibition, which is succeeded by tissue growth (64, 110). In laboratory rodents specifically, estrogens stimulate the uterus to grow rapidly and vigorously, especially so since their estrous cycle lasts about 4 days. Hence, the rat uterus is appropriate for screening for estrogen agonists and antagonists. Following successful ovariectomy, reduced uterine weight would be expected in the OVX group, as early as 1 to 3 weeks postoperatively, and in our study of 6 months, the uterus did weigh significantly more in the control group (104). A statistically significantly higher mean uterine weight of the OVX+CS group in our results would indicate a positive response to *C. siliqua* but that was not the case, neither for its absolute weight nor its relative weight (64). *Ceratonia siliqua* was found not to affect the weight of the gastrocnemius muscle, as well.

A statistically significant difference among the three groups was noted for abdominal fat and brain weight. As anticipated, these tissues weighed significantly more in the OVX group compared to the OVX+CS group, but their weights were alike for control and OVX+CS groups. Perhaps the lack of estrogens modifies adipose tissue metabolism and abdominal fat distribution (111). The relative abdominal fat weight was also significantly higher in the OVX group but did not differ between the control and OVX+CS groups. These results concur with those of Fujita *et al.* showing that *C. siliqua* may be used against obesity since carob pod polyphenols suppressed the differentiation of adipocytes through the posttranscriptional regulation of CCAAT enhancer binding protein beta (112). The relative weights of the heart and brain were unaffected by *C. siliqua* in our study. Nonetheless, the methanolic extract of carob pods has been found to prevent the short-term memory impairment in rats caused by chronic stress, probably due to inhibiting the decrease in the level of brain-derived neurotrophic factor in the hippocampus (113). Moreover, pre- and post-treatment with aqueous carob extract ameliorated the effects of amiodarone (in a lung toxicity model in rats), and waterpipe smoke exposure (passive smoking) on the brain and lung (32), probably because of its antioxidant impact.

Apart from the 6-month duration of the study, the time of beginning *C. siliqua* administration may be considered another limitation of this study to consider. During early menopause, bone may be less responsive to nutritional intervention due to hormonal alterations (114). Moreover, the guideline of the Organization for Economic Co-

operation and Development (OECD) for the testing of chemicals in rodents for estrogenic properties refers that substance administration in rats should begin not less than 2 weeks after ovariectomy so that the uterus will have regressed. In addition, at least two dose groups are recommended with administration for more than 3 days as this duration of administration surpasses the uterine response time to endogenous estrogens, and weakly active substances may be more easily recognized. Frequency of administration, *C. siliqua*'s pharmacokinetics, means of administration, and dosage *per se* are other points for consideration. The possible exposure of the animals to other estrogens was not investigated. For example, the bedding (for example corncob demonstrates anti-estrogenic effects), or the cage material (animals maintained in polycarbonate and polysulfone cages, especially for old cages, are exposed to bisphenol A, a monomer with estrogenic activity), should also have been examined. Finally, according to the OECD, the absence of estrous should have been confirmed by the observation of epithelial cells swabbed from the rat vaginas, for example, 10-14 days after ovariectomy, for at least 5 consecutive days (64, 115, 116), which was not considered during our study.

Conclusion

Ceratonia siliqua exerts beneficial effects on proximal tibial BMD and abdominal fat. Nevertheless, *C. siliqua* failed to improve biomechanical strength in the femoral diaphysis during the three-point bending test. Further research is required, for example regarding the *C. siliqua* administration period, its dosage, and frequency of administration, in order to determine the efficacy of *C. siliqua* as an osteoprotective agent.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

Authors' Contributions

The Authors of this article made the following contributions: AAN, DG, AG, AEP, KS, AZ, SZ, SKK, EDP, EC, GK, TK and IAD contributed to project design and data analysis; AG to statistical analysis; AAN, DG, AG, SKK, GK, IAD and TK to the final review, article presentation and critical review of the article for important intellectual content; AAN, DG, AEP, AZ, SZ, EDP and IAD to the conduct of experiments and laboratory tests; and SM, DM and AV to chow preparation.

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