

Original Article

Possible antiosteoporotic mechanism of *Cicer arietinum* extract in ovariectomized rats

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Abstract: Objective: The present study aimed to throw the light on the anti-osteoporotic mechanism of *Cicer arietinum* extract (CAE) seeds against ovariectomized (OVX) rats. Methods: Seventy female rats were divided into two groups. The first group (14 rats/group) represented normal rats (Sham operated) while the second group (56 rats/group) underwent bilateral ovariectomy (OVX). After one week of recovery from ovariectomy surgery, the second group was randomly subdivided into 4 subgroups (14 rats/ each subgroup). The rats administered orally; distilled water (vehicle) (1st subgroup), *Cicer arietinum* extract (CAE) (500 or 1000 mg/kg body weight/day) (2nd and 3rd subgroups), alendronate (6.5 mg/kg mg/kg body weight) as a positive control one time/week (4th subgroup), daily for 10 weeks. Results: The present study demonstrated that ovariectomy caused significant decrease in bone mineral density (BMD) and content (BMC), Bone-specific alkaline phosphatase (BALP), calcium (Ca), phosphorus (P), parathyroid hormone (PTH) and calcitonin levels. Furthermore, ovariectomy induced significant elevation of tartrate-resistant acid phosphatase 5b (TRAP 5b) and receptor activator of nuclear factor (NF-kappa β) ligand (RANKL) concentration. Conversely, osteoprotegerin (OPG) and OPG/RANKL ratio were decreased following ovariectomy. The present work suggests that CAE has antiosteoporotic action against ovariectomy effects and its activity may result from its phytochemical and/or phytoestrogen contents. Conclusion: The ongoing study speculates that the CAE exerts its action through regulation of RANK/RANKL/OPG system. As, CAE not only promotes osteoblast differentiation, but also up-regulates OPG and downregulates RANKL secretion in osteoblasts, subsequently prevents bone loss and osteoporosis.

Keywords: *Cicer arietinum*, osteoporosis, ovariectomy, bone remodeling regulators, bone resorption marker.

Introduction

Osteoporosis is often called a silent disease of aging because bone loss occurs without symptoms until microarchitectural deterioration and bone fracture occurs [1]. The most common type of osteoporosis is the bone loss associated with estrogen deficiency in postmenopausal women [2]. Osteoporosis has become a major health hazard disease in recent years, afflicting over 2000 million people worldwide [3]. In Egypt, the statistics estimated the prevalence of osteoporosis in 2011 as about 8 million cases out of 80 million populations [4]. Thus, the management of osteoporosis and its complications is a socioeconomic priority [5].

Antiresorptive agents such as estrogen and bisphosphonates are considered the major conventional therapy for postmenopausal

osteoporosis [6]. Unfortunately, recent evidence indicates that the long term use of estrogen treatment (estrogen replacement therapy) is accompanied by side effects such as increased risk of breast, ovarian and endometrial cancers [7]. Moreover, use of bisphosphonates is associated with esophageal cancer, gastrointestinal and osteonecrosis of the jaw [8]. Therefore, there is an urgent demand to search for potential non-pharmacological alternative therapy for osteoporosis.

Phytoestrogens are diverse groups of natural estrogenic compounds found in a variety of plants and in their seeds [9]. Reliable evidence has indicated that phytoestrogens offer the best potential therapy for menopausal women because they are safe and mimic the endogenous estrogen structure [10]. The bioactivity of these compounds is based on their structural

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similarity with 17 β -estradiol and their ability to bind to estrogen receptors [11]. Leguminosae are the most important family with regard to the content of isoflavones (the major component of phytoestrogen) [12].

Most of the previous studies neglecting the effect of the third most important grain legume in the world on the basis of total grain production, according to FAO [13] which is the chickpea "*Cicer arietinum*". The chickpea is a member of the cool season Fabaceae (Leguminosae) family and considered as a good starting material in the food industry because of its protein quality in comparison with that of the soybean [14]. In Egypt, chickpea seeds are usually consumed at the raw green and tender stage (unripe stage), called Malana, or in the form of mature dry seeds after parching as a popular snack food and thereby it is widespread and inexpensive [15].

The discovery of the factors involved in the control of osteoclasts, and hence osteoporosis, has moved bone research into a new era. Some of these factors are the receptor activator of nuclear factor- κ B ligand (RANKL) and osteoprotegerin (OPG) which considered fundamental factors that control osteoclast formation and activation [16]. This process is self-regulated by the production of the soluble endogenous antagonist OPG, which essentially acts as a decoy for RANKL, preventing its binding to RANK receptor.

Therefore, the ongoing study was carried out to investigate the effect and the mechanism of *Cicer arietinum* seed extract (CAE) on bone turnover markers in ovariectomised rats, an excellent model of menopause and a well-established animal model for the study of postmenopausal osteoporosis.

Materials and methods

Plant material

Seeds of chickpea (*Cicer arietinum* L.) cultivar Giza 1 were purchased from the Agricultural Research Center of Giza, Egypt.

Preparation of Cicer arietinum extracts (CAE)

Methanol is considered the best organic solvents that dissolve the majority of phytochemicals and/or phytoestrogens [17]. One gram of finely ground dry *Cicer arietinum* seeds was

mixed with 4 ml of methanol, heated at 60°C for 1 h while being shaken in a water bath. The resulting extract was centrifuged at 10000 rpm, 5°C for 20 min. The resulting supernatant was filtered through filter paper (Whatman number 1), concentrated on rotary evaporator and dried by lyophilizer apparatus (LABCONCO lyophilizer, shell freeze system, USA). The prepared CAE was stored in desiccator until use.

Determination of active compounds of Cicer arietinum extract (CAE)

Phytochemical screening of CAE: Qualitative tests were used for the detection of the carbohydrates and/or glycosides, saponin, phytosterols, terpenoids, flavonoids, alkaloids and tannins in CAE according to the methods adopted by Trease and Evans [18]; Parekh and Chanda [19]; Ayoola et al. [20] and Kodangala et al. [21].

Estrogenic contents of CAE

Further investigation was carried out by HPLC system to search for the active estrogenic components founded in CAE according to Griffith and Collision [22].

Animals

Adult female Wistar albino rats (*Rattus norvegicus*) weighing 150-170 g were obtained from the animal house of the National Research Center (NRC), Egypt. Rats were housed in polypropylene cages in air-conditioned room at a temperature of 23 \pm 2°C and under natural day and night cycle. They were fed standard chow pellets and drinking water *ad libitum*. The rats were kept for a week before the commencement of the experiment for acclimatization. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Science, Cairo University (CUFS-F-PHY-03-13). All the experimental procedures were carried out in accordance with international guidelines for the care and use of laboratory animals.

Acute toxicity test (OECD guideline 425 adoption)

The acute oral toxicity test was done according to the organization for economic cooperation and development (OECD) based on acute oral toxicity-up and down procedure 425 guideline

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[23]. In this study, the rats ($n = 5$) were fasted overnight and administered for onetime the tested substance orally at a limit dose of 5000 mg/kg body weight via gastric gavage and euthanized after 14 days. The LD_{50} was predicted to be above 5000 mg/kg if three of five rats or more were survived. In the present study, ten healthy female albino rats were randomly divided into two groups of equal size (5 rats/each group).

Group (1): Served as a standard control group and administered distilled water.

Group (2): Administered *C. arietinum* extract (CAE) as suspension in distilled water.

Group 1 (control group) was given distilled water equivalent to the volume administered to the other tested experimental groups. Group 2 administered CAE at a dose of 5000 mg/kg body weight daily for 2 weeks. Body weights were estimated periodically at 0, 7 and 14 days of administration and the final body weight change was calculated at the end of the study. Furthermore, general observation such as pilo-erection, salivation and lacrimation was performed at 0 min, 30 min, 1, 2, 4, 6 h and thereafter every day for 14 days to check abnormal clinical manifestation and mortality. On day 15, all survived rats were euthanized and the liver and both kidneys were excised, weighed and fixed in 10% formalin for 24 h. Liver and kidneys were processed and stained with hematoxylin and eosin stain for histopathological examination [24]. The supposed median effective dose (ED_{50}) of a forementioned CAE powder was tenth of the LD_{50} , i.e. 500 mg/kg body weight.

Experimental design

Ovariectomy process was carried out according to [25]. Seventy female rats were divided into two groups. The first group (14 rats/group) represented normal rats (Sham operated) while the second group (56 rats/group) underwent bilateral ovariectomy (OVX) under light diethyl ether [26]. The rats in the Sham-operated group were anesthetized, laparotomized, and sutured without removing their ovaries. After one week of recovery from ovariectomy surgery, the second group was randomly subdivided into 4 subgroups (14 rats/each subgroup). The first OVX subgroup was administered orally distilled water (vehicle) daily for 10 weeks. The other two OVX subgroups were administered orally *Cicer arietinum* extract (CAE) (500 or

1000 mg/kg body weight/day) for 10 weeks. The fourth OVX subgroup treated orally with alendronate, suspended in distilled water, as a positive control one time/week (for 10 weeks) with a dose of 6.5 mg/kg mg/kg body weight. Meanwhile, rats were administered distilled water during the remaining days of the week [27].

Animals handling

At the end of the tenth week, half of the experimental rats/each group was anesthetized for assessment of bone mineral density (BMD) and bone mineral content (BMC) of the total skeleton, femur and tibia. While the other half was euthanized and blood samples of each group were collected in centrifuge tubes without anticoagulant. Left femora were excised and the surrounding tissues were removed then washed in cold saline and stored at -80°C until used.

Serum preparation

The collected blood was centrifuged at 3000 rpm for 20 minutes and the collected sera were stored at -80°C until use.

Bone homogenate preparation

The left femora/each group were ground and about 100 mg was taken and homogenized with 2 ml of 0.1 M Tris- HCl buffer (pH 7.2). The homogenate was centrifuged at 3000 rpm for 30 min. at 4°C and the supernatant was stored at -80°C until use [28].

Measurement of bone mineral content (BMC) and bone mineral density (BMD)

The BMD and BMC of the total skeleton, femur and tibia were estimated in anesthetized rats using dual energy X-ray absorptiometry (DEXA, Norland \times 46, Version 3.9.6). Instrument equipped with dedicated software for small animal measurements. BMC (expressed in grams) was divided by the area of the site that was scanned to obtain BMD [expressed in grams per centimeter square] [29].

Determination of bone formation markers

Serum calcium (Ca) and phosphorus (P) concentrations were estimated according to the method described by Gindler and King [30] and El-Merzabani *et al.* [31], respectively. Additionally, serum parathyroid hormone (PTH) and calcitonin levels were estimated through radioim-

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Table 1. Phytochemical screening of *Cicer arietinum* extract (CAE)

Test	Observation	Result
1. Tests for glycosides and/or carbohydrates		
a) Molish's test	Appearance of a violet ring at the junction of sulphuric acid and filtrate	Presence of carbohydrates and/or glycosides
b) Benedict's test	No appearance of red precipitate	Absence of reducing sugars
		This result indicates the presence of polysaccharides and different types of glycosides
2. Tests for saponins		
Froth test	A voluminous persistent froth	Presence of saponin
3. Test for phytosterols		
Liebermann-Burchard test	Appearance of reddish violet color at the junction of two layers and a bluish green color in the acetic layer	Presence of phytosterols
4. Test for terpenoids		
Salkowski's test	Appearance of red color	Presence of terpenoids
5. Test for flavonoids		
Sodium hydroxide test	The appearance of yellow color	Presence of flavonoids
6. Test for alkaloids and/or basic nitrogenous substances		
Wagner's test	Brown precipitate	Presence of alkaloids
7. Test for resins		
	Negative	Absence of resins
8. Test for tannins		
Lead acetate	White precipitate	Presence of tannins

radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA), respectively according to the method described by Deftos [32].

Bone-specific alkaline phosphatase (BALP) was determined in bone supernatant using Bio-diagnostic kit [33]. Moreover, bone Ca and P contents were determined according to the method adopted by Teófilo *et al.* [34].

Determination of bone resorption marker and bone remodeling regulators

Tartrate-resistant acid phosphatase 5b (TRAP 5b), RANKL and OPG were estimated in serum by enzyme linked immunosorbent assay (ELISA) and the OPG/ RANKL ratio was calculated.

Statistical analysis

Statistical analysis was carried out using SPSS v. 15 software. All data were expressed as means \pm standard error of mean (SEM). The data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan test to determine difference between groups. *P* values of less than 0.05 were considered statistically significant.

Results

Phytochemical screening of CAE

The phytochemical screening of *C. arietinum* extract (CAE) showed different active constituents (**Table 1**). These are glycosides and/or carbohydrates, saponins, phytosterols, terpenoids, flavonoids, alkaloids and/or basic nitrogenous substances, and tannins.

Phytoestrogenic compounds of CAE

Figure 1 demonstrates the presence of some estrogenic compounds in the methanolic extract of *Cicer arietinum* seeds which are daidzein, genistein, formononetin and biochanin A, (the four main components of isoflavones).

Acute toxicity test

The oral administration of *Cicer arietinum* extract (CAE, 5000 mg/kg body weight) caused neither mortality nor any sign of clinical abnormality for two weeks post administration. Regarding the body weight, the body weight changes, throughout the two weeks, in the

treated group was found more or less similar to that of the control group. Moreover, no significant changes in the organs weight (liver and kidneys) were recorded in CAE treated rats when compared to the control ones. Furthermore, histological examination illustrated that the administration of CAE did not induce gross pathological changes, since the liver architecture of CAE treated rats is more or less similar to the normal liver architecture (**Figure 2**). Thus, from the aforementioned results; one can conclude that the LD₅₀ of CAE powder is surely more than 5000 mg/kg body weight.

BMD and BMC of the total skeleton, femur and tibia

BMD of total skeleton, femoral and tibial bones were decreased significantly ($P < 0.05$) in the OVX rats than the corresponding ones of the sham rats. On the other hand, treatment with CAE (500 mg/kg body weight) increased the BMD levels, but this increase was statistically significant ($P < 0.05$) only in case of the tibial bone. Meanwhile, the administration of CAE (1000 mg/kg body weight) significantly modulating ($P < 0.05$) the reduction in BMDs caused by ovariectomy in all studied bones (**Figure 3**). On the contrary, BMD values of all selected bone regions were found significantly higher ($P < 0.05$) than those of untreated OVX rats, subsequent to alendronate administration (**Figure 3**).

BMCs of total skeleton, femur and tibial regions were significantly lower ($P < 0.05$) in the OVX group than in the sham group (**Table 2**). Treatment with CAE (500 mg/kg body weight) increased the BMC values significantly ($P < 0.05$) in all measured bones, while treatment with higher dose of CAE(1000 mg/kg body weight) only increased significantly ($P < 0.05$) the total skeleton and tibial BMCs, as compared to OVX rats (**Table 2**). The obtained results also revealed that significant elevation ($P < 0.05$) was recorded in the total skeleton and femoral BMCs after administration of alendronate, as compared to OVX group (**Table 2**).

Bone formation markers

Figures 4-7 indicate that ovariectomy induced a significant decrease ($P < 0.05$) in the BALP activity, calcium and phosphorus contents of serum and tibial bone and serum calcitonin

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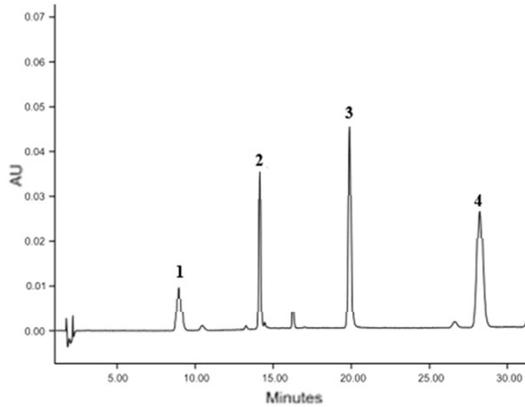


Figure 1. HPLC chromatogram of methanolic extract of *Cicer arietinum* (CAE) seeds. (1) Daidzein, (2) Genistein, (3) Formononetin and (4) Biochanin A.

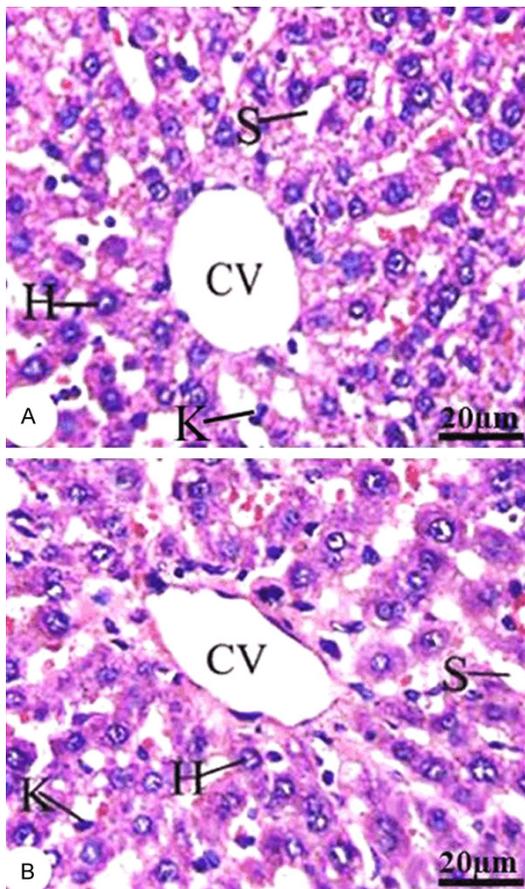


Figure 2. Photomicrographs of liver sections stained by hematoxylin and eosin showing the effect of *Cicer arietinum* (CAE) at an acute limit dose (5000 mg/kg body weight) on liver architecture. A. Control group, showing the normal liver architecture with distinct hepatocytes (H), sinusoidal spaces (S), central vein (CV), kupffer cells (K), well preserved cytoplasm and prominent nuclei. B. CAE group, showing almost normal liver architecture. Scale bar = 20 µm.

level, as compared to the corresponding Sham rats. Conversely, **Figure 7** revealed that PTH concentration in the ovariectomized rats was significantly higher ($P < 0.05$) than the corresponding one of the Sham group. Treatment of OVX rats with CAE (500 mg/kg body weight) significantly ($P < 0.05$) increase the BALP activity and tibial calcium level. However, post-treatment with CAE (1000 mg/kg body weight) significantly increased BALP activity and serum P content when compared to OVX rats (**Figures 4, 6**). On the other hand, level of PTH decreased significantly ($P < 0.05$) following CAE treatment at the two tested doses (**Figure 7**). Regarding to the effect of the alendronate treatment, the obtained results showed a non-significant change in the BALP, serum Ca, tibial P and serum calcitonin levels subsequent to alendronate administration, as compared to OVX group (**Figures 4-7**). As compared to OVX group, alendronate administration caused significant increase ($P < 0.05$) in the tibial calcium and serum P concentrations. On the other hand, treatment of OVX rats with alendronate reduced the serum PTH significantly ($P < 0.05$).

Bone resorption marker and bone remodeling regulators

Ovariectomy process caused a significant increase ($P < 0.05$) in the levels of TRAP 5b and RANKL, while, the OPG concentration and OPG/RANKL ratio significantly decreased ($P < 0.05$), as compared to Sham control rats (**Table 3**). Treatment with CAE (500 and 1000 mg/kg body weight) attenuates significantly ($P < 0.05$) the elevation of TRAP 5b level induced by ovariectomy (**Table 3**). Meanwhile, the administration of CAE at the two tested levels ameliorated the elevation of RANKL level induced by ovariectomy, however, this effect was statistically significant ($P < 0.05$) only at the low dose. In contrast, administration of CAE at the two selected doses significantly elevated ($P < 0.05$) OPG concentration and OPG/RANKL ratio observed in OVX rats (**Table 3**). However, here is no significant change in the RANKL and OPG levels as well as the OPG/RANKL ratio subsequent to alendronate administration, as compared with OVX group (**Table 3**).

Discussion

Osteoporosis is considered as a major health problem; especially in Egypt, wherever Egyptian

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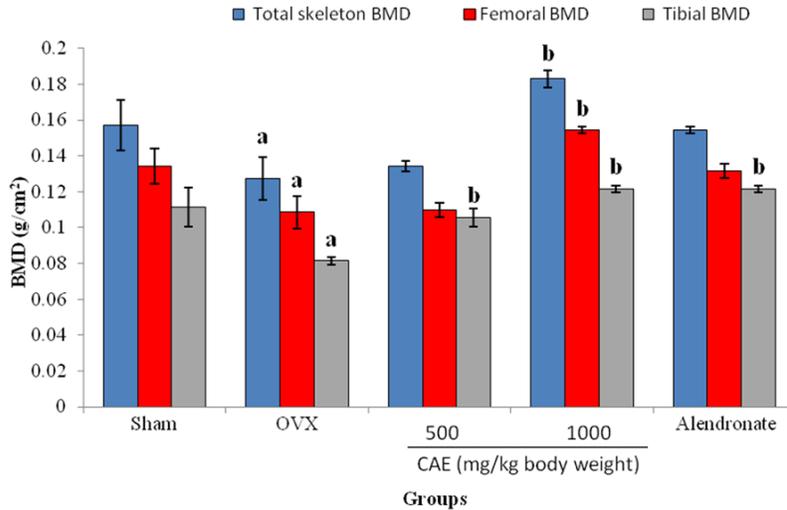


Figure 3. Effect of *Cicer arietinum* (CAE) on bone mineral density (BMD) in ovariectomized rats. Values are mean \pm SEM (n = 7). a: significant ($P < 0.05$) as compared with Sham group. b: significant ($P < 0.05$) as compared with OVX group.

women have generally lower bone mineral density compared to women in western countries [35]. In Egypt 6.5% of females aged 20 years and above suffers from osteopenia and about 10-13% of postmenopausal women suffer from osteoporosis [4]. Phytoestrogens are a group of biologically active plant substances with a chemical structure similar to that of estradiol, an endogenous estrogen [36]. There is a growing demand for the discovery of the new phytoestrogenic source to be used as safe and effective hormonal replacement therapy. The ongoing study was designed to study the efficacy of *Cicer arietinum* (CAE) seeds extract to simulate the conditions under which this extract would be utilized to treat osteoporosis *in vivo*. Recently, several studies have been evaluated the estrogenic activity of isoflavones extracted from CAE *in vitro* and *in vivo* [37, 38]. In consistent with their reports, the present investigation proved that the CAE contains isoflavones [daidzein, genistein, formononetin and biochanin A]. The ovariectomized rat (OVX) is an excellent preclinical animal model that correctly emulates the important clinical feature of the estrogen depleted human skeleton and the response of therapeutic agents [39, 40].

Measurement of bone mineral density (BMD) and bone mineral content (BMC) using dual-energy X-ray absorptiometer (DEXA) is considered as a golden standard assessment for the evaluation of individuals at risk for osteoporosis,

as it best predicts the fracture risk in people without previous fractures [41, 42]. Inconsistent with Ikeda *et al.* [43], the present investigation revealed that ovariectomy significantly reduced the total skeleton, femoral and tibial BMDs and BMCs. It is well known that calcium (Ca) and phosphorus (P) are widely accepted as markers for bone formation [44]. Inconsistent with the finding of Saleh and Saleh, [45] and Wahba & Al-Zahrany [44], the present study revealed that ovariectomy led to a significant decrease in both calcium and phosphorus in

the serum and tibial bone. So, the decrease in BMD and BMC following ovariectomy in the present study may be due to the incomplete mineralization of bone that results from calcium and phosphorus deficiencies as well as decreased calcium absorption. Burali *et al.* [46] reported that estrogen deficiency is associated with impaired calcium balance and thereby reduced calcium absorption as well as increased renal excretion of calcium. Meanwhile, Hassan *et al.* [47] reported that ovariectomy has been shown to alter phosphate homeostasis and lead to significant decrease of its level. Administration of CAE supplement improved BMD, BMC and the loss of tibial Ca content caused by ovariectomy. It was reported that estrogen and in turn compounds that mimic estrogen enhance calcium absorption by up-regulating the intestinal Ca transporter genes [24, 47]. Interestingly, the current study suggested that CAE has a favorable effect on Ca absorption and its deposition in bone, which may contribute to increase the BMD and BMC. Again, the ameliorative effect of CAE on BMDs and BMCs of the OVX rats may be due to its genistein and daidzein contents recorded in the present study. Similarly, Ghanem [48] mentioned that genistein and daidzein have a beneficial role in preventing bone loss and increasing bone formation, respectively. The present study disclosed that alendronate treatment improved BMD and BMC of the studied bones of OVX rats. These results are in agreement

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Table 2. Bone mineral content (BMC) of different bone regions of the different experimental groups of rats

Bone region	Sham	OVX			Alendronate
		Vehicle	CAE (mg/kg body weight)		
			500	1000	
Total skeleton	8.43 ± 0.923 ^a	1.18 ± 0.081 ^b	5.05 ± 0.173 ^c	3.22 ± 0.194 ^d	4.04 ± 0.538 ^{c,d}
Femur	0.48 ± 0.08 ^a	0.05 ± 0.017 ^b	0.15 ± 0.002 ^c	0.11 ± 0.006 ^{b,c}	0.19 ± 0.029 ^c
Tibia	0.29 ± 0.035 ^a	0.12 ± 0.007 ^b	0.20 ± 0.006 ^{c,d}	0.28 ± 0.008 ^a	0.11 ± 0.009 ^b

Values are mean ± SEM (n = 7). Values with different superscript letters are significantly different (P < 0.05).

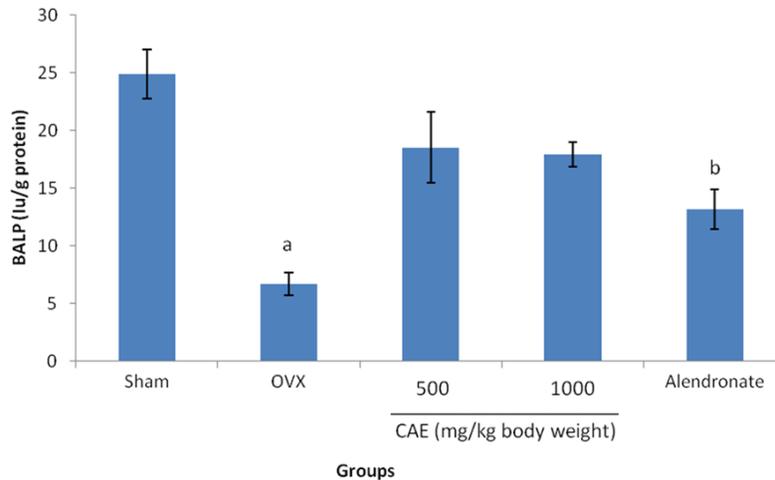


Figure 4. Effect of *Cicer arietinum* (CAE) on Bone-specific alkaline phosphatase (BALP) activity in ovariectomized rats. Values are mean ± SEM (n = 7). a: significant (P < 0.05) as compared with Sham group. b: significant (P < 0.05) as compared with OVX group.

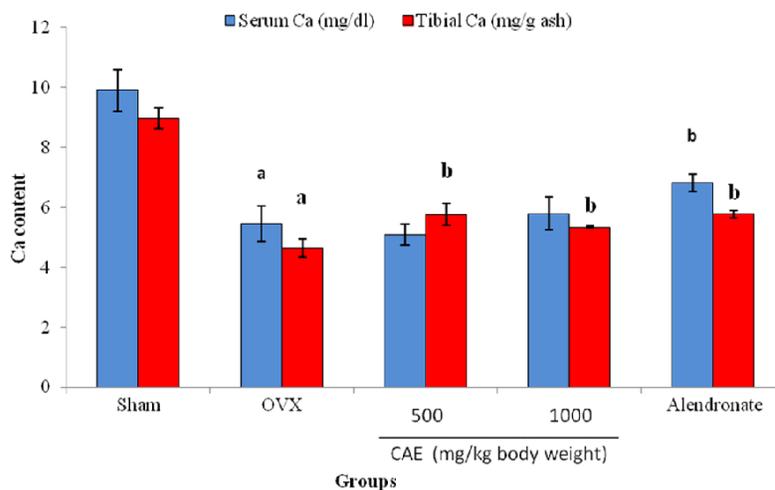


Figure 5. Effect of *Cicer arietinum* (CAE) on serum and tibial Ca in ovariectomized rats. Values are mean ± SEM (n = 7). a: significant (P < 0.05) as compared with Sham group. b: significant (P < 0.05) as compared with OVX group.

with the results of Ikeda et al. [43] and McClung et al. [49] explained that alendronate

increase BMD by increasing matrix mineralization. Moreover, the increase in bone calcium content after alendronate treatment revealed that it had no deleterious effect on mineralization according to Balena et al. [50] and Azuma et al. [51].

Bone specific alkaline phosphatase (BALP) is associated with osteoblast activity and its level has been used to assess bone formation [42]. The present study revealed that BALP activity decreased significantly in OVX rats. In consonance with the report of Sontakke and Tare [52], the present decrease in the activity of BALP may be due to the disturbance in osteoblastic function and/or an imbalance between osteoclastic and osteoblastic activities, in which the former supersedes the latter. The current study demonstrated an ameliorative effect of CAE on BALP activity in OVX rats. This action may be due to the flavonoids, saponins, daidzein and genistein contents of CAE. Similarly, Zhang et al. [53] mentioned that total flavonoids exhibited an improvement in the development of osteoblasts by promoting the alkaline phosphatase. Furthermore,

Cvoro et al. [54] stated that saponins promoting osteoblast proliferation and hence increase

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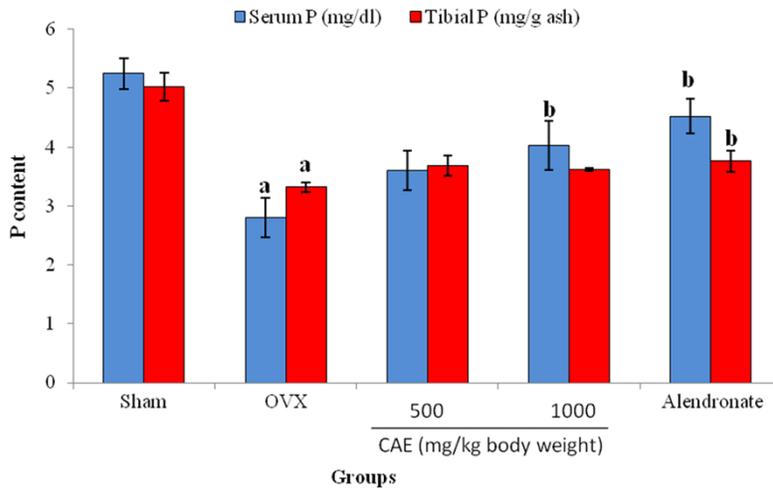


Figure 6. Effect of *Cicer arietinum* (CAE) on serum and tibial P in ovariectomized rats. Values are mean \pm SEM (n = 7). a: significant ($P < 0.05$) as compared with Sham group. b: significant ($P < 0.05$) as compared with OVX group.

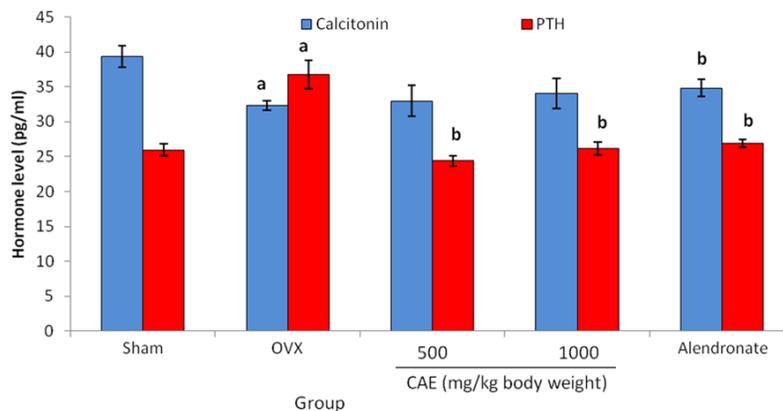


Figure 7. Effect of *Cicer arietinum* (CAE) on serum calcitonin and parathyroid hormones (PTH) levels in ovariectomized rats. Values are mean \pm SEM (n = 7). a: significant ($P < 0.05$) as compared with Sham group. b: significant ($P < 0.05$) as compared with OVX group.

the BALP activity. In addition, Shirke *et al.* [55] added that daidzein and genistein have been found to have stimulatory effects on protein synthesis and on ALP released by osteoblast cells *in vitro*. Shirke *et al.* [56] reported that alendronate considered as an antiresorptive drug and has no relation with bone formation. This supports the current result of alendronate, since it did not affect the BALP level, as compared to untreated OVX rats. Shirke *et al.* [56] reported that alendronate considered as an antiresorptive drug and has no relation with bone formation. This supports the current result of alendronate, since it did not affect the

BALP level, as compared to untreated OVX rats.

It is known that parathyroid hormone (PTH) and calcitonin (CT) regulate serum calcium and inorganic phosphate concentrations [57]. In conjunction with the report of Canpolat *et al.* [58], the present results revealed a significant elevation in PTH level and a significant reduction in calcitonin level of OVX rats. Prabhakara Reddy and Lakshmana [59] attribute the hyperparathyroidism in osteoporotic rats to calcium deficiency and exacerbated by estrogen deficiency. The current study suggests that estrogen deficiency leads to decreased calcium level in blood, which ultimately increases the PTH concentration, where calcium is the major regulator of PTH secretion [60]. Administration of CAE modulates the level of PTH but has no effect on calcitonin level and this is agreeing with the finding of Yilmaz *et al.* [57] and Shalaby *et al.* [10]. PTH reduction subsequent to CAE would indicate a reduction of bone resorption and osteoclastic activities [61].

The present finding showed that CAE failed to restore the completely calcitonin level, which, may indicate that both of selected doses were not the optimal and effective dose for restoring the calcitonin level. Similarly, Li and Yu [62] concluded that isoflavone, one of the CAE constituents, didn't influence the level of calcitonin. With respect to alendronate treatment, it alleviated the increased PTH level caused by ovariectomy. The current study hypothesized that the inhibition of bone resorption caused by alendronate may cancel the hyperparathyroidism activity, this suggestion is in line with Rodan [63]. Conversely, alendronate has no

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Table 3. Effect of *Cicer arietinum* (CAE) on different osteoporotic biochemical markers in ovariectomized rats

Group	Parameter	TRAB 5b (pg/L)	RANKL (pmol/L)	OPG (ng/L)	OPG/RANKL ratio
Sham		483.53 ± 25.75 ^{a,c}	41.25 ± 7.80 ^a	359.52 ± 19.06 ^a	0.55 ± 0.11 ^a
OVX		651.98 ± 41.46 ^b	108.32 ± 15.07 ^b	239.25 ± 14.52 ^b	0.10 ± 0.02 ^b
500	CAE (mg/kg	539.88 ± 16.57 ^c	67.52 ± 8.76 ^{a,c}	281.02 ± 3.81 ^c	0.32 ± 0.007 ^{c,d}
1000	body weight)	487.30 ± 26.74 ^{a,c}	88.70 ± 14.38 ^{b,c}	293.08 ± 11.12 ^c	0.32 ± 0.04 ^{c,d}
Alendronate		558.93 ± 31.55 ^c	121.48 ± 16.03 ^b	270.38 ± 4.90 ^{b,c}	0.13 ± 0.022 ^{b,c}

Values are mean ± SEM (n = 7). Values with different superscript letters are significantly different (P < 0.05). TRAP 5b: serumtartrate-resistant acid phosphatase 5b level, RANKL: serumreactive activator nuclear factor κβ ligand, OPG: osteoprotegerinand OPG/RANKL ratio.

effect on the calcitonin level. This finding was inconsistent with Shirke *et al.* [56] who stated that alendronate has no bone formation activity.

Circulating serum tartrate-resistant acid phosphatase (TRAP) 5b is solely of osteoclastic origin [64]. Therefore, its serum concentrations have been found to correlate directly with the number of osteoclasts in bone biopsies, making it a valuable marker of bone resorption [65]. In conjunction with the report of Karmakar *et al.* [66], the current study revealed that TRAP 5b increased significantly in OVX rats. This increase may be due to estrogen deficiency since, TRAP 5b level correlated inversely with estrogen level [65]. The current finding showed that, CAE attenuates the increased serum TRAP 5b level induced by ovariectomy reflects a decrease in osteoclast activity [67]. Therefore, the ongoing study proved that CAE supplement may play an important role prevent the induction of bone resorption associated with the estrogen deficiency in OVX rats. Moreover, the current study backed the ameliorative effect of CAE on TRAP 5b to its flavonoids, saponin and alkaloid contents. This suggestion is confirmed by the findings of Chiba *et al.* [68]; Nian *et al.* [69] and Hu *et al.* [70]. Treatment with alendronate, in the current study, resulted in decreased the serum TRAP 5b level. von Rosenberg *et al.* [27] reported that alendronate treatment blocks the osteoclast activity and thus reduces bone resorption. In addition, Katz and Weirnerman [71] clarified the antiresorptive mechanism of alendronate. It is interesting to suggest that the CAE and/or CES treatment was better than alendronate drug treatment, as they increased the BALP activity and decreased the TRAP 5b activity; but alendronate decreased

the TRAP 5b activity only, as compared to untreated OVX rats.

Bone remodeling requires a balance in the activity of the osteoblasts and the osteoclasts. To investigate the potential mechanism involved in the osteocurative effects of CAE, the triad RANK/ RANKL/OPG system was studied. The discovery of the RANK/RANKL/OPG pathway provided new mechanistic insights into the pathogenesis of bone loss that accompanies estrogen deprivation [72]. In consonance with the finding of Zhang *et al.* [73] and Shalaby *et al.* [10] the present study showed that the OVX group has lower OPG value and significant decrease in the OPG/RANKL ratio, as compared with sham group which may be due to estrogen deficiency. Viewed in conjunction with the report of Rahman *et al.* [74], the stimulation of osteoclast differentiation subsequently OPG level reduction, is one of different mechanisms by which estrogen deficiency cause bone loss. Moreover, the increased PTH level of OVX group, in the present study, may be the causative factor for increasing the RANKL level and decreasing the OPG level [75].

Interestingly, the present supplementation of CAE to OVX rats increased the OPG level. Hashish *et al.* [76] disclosed that enhancement of OPG level decreases the interaction of RANKL with RANK which consequently reduce osteoclastic bone resorption. Moreover, similar to the present study, Zhang *et al.* [73] and Shalaby *et al.* [10] reported decreased RANKL level and increased the OPG/RANKL ratio following phytoestrogen supplementation. Furthermore, Hu *et al.* [70] clarified that alkaloids attenuate osteoclast differentiation and function through inhibition of RANKL in osteoblast

cells. It seems that the ameliorative effect of CAE on osteoclastogenesis may be due to its phytoestrogenic and alkaloid contents. Thereby, the present result expected that CAE treatment inhibit differentiation of osteoclasts by stimulating OPG and inhibiting RANKL production in osteoblasts. Subsequently, the CAE supplement may have antiresorptive effect and this suggestion supported by Hashish *et al.* [76], who mentioned that OPG markedly inhibit bone resorption. The current study revealed that alendronate has not any statistical effect on the RANKL and OPG levels. This result was found inconsistent with the result of Kim *et al.* [2] and Boyce and Xing [77]. They mentioned that alendronate inhibit osteoclast formation and bone resorption via a direct effect on osteoclasts. Interestingly, the present finding suggested that the present supplements (CAE and CES) were better than alendronate drug, since they enhance OPG and reduce RANKL expression and consequently impair osteoclastogenesis and prevent bone loss in OVX rats.

In conclusion, based on the results of the present investigation, CAE stimulates OPG synthesis and reduces RANKL expression in osteoblasts, resulting in an increase of the OPG/RANKL ratio and decrease of osteoclast differentiation. These results suggested that CAE not only promotes osteoblast differentiation, but also up-regulates OPG and downregulates RANKL secretion in osteoblasts, subsequently prevents bone loss and osteoporosis. Moreover, genistein and daidzein could be responsible for the CAE antiosteoporotic activity.

Disclosure of conflict of interest

None.

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