

Ameliorative Effects of *Cicer arietinum* Extract and *Coelatura aegyptiaca* Shell Powder on Estrogen Sensitive Organs in Ovariectomized Rats

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Abstract: Postmenopausal osteoporosis has become a social problem as it gives rise many health-related problems, therefore osteoporosis disease requiring appropriate management strategies. Replacement therapy is effective for both prevention and treatment, but recent findings have shown that its long term administration is not as safe as was previously thought, thereby alternative treatments are urgently needed. The current work selects *Cicer arietinum* extract (CAE) as one of most important legume and *Coelatura aegyptiaca* shell (CES) powder as calcium source to study their effectiveness against osteoporosis through their effects on estrogen determinant organs. The present study revealed that CAE and/or CES decline the body weight gain induced by ovariectomy (OVX). Furthermore, CAE and/or CES improve femur and tibial weights changed by OVX. Additionally, they ameliorated the abnormal weights of uterus, vagina and thymus caused by OVX. The ongoing study concluded that the concurrent treatment of CAE and CES may be effective in treating osteoporosis, as evident by their amelioration on estrogen sensitive organs.

Key words: Legume • Calcium source • Ovariectomy • Estrogen-determinant organs

INTRODUCTION

Bone is a specialized living connective tissue that makes up the skeletal system. Throughout life, bone is formed and resorbed in a dynamic process for renewal of bone tissue, called "bone remodeling". This process undergoes in a continuous cycle of active bone formation (through the activity of osteocytes and osteoblasts) and bone resorption (through the osteoclasts). Several factors along with aging have been shown to be risk factors in the etiology of osteoporosis. Particularly; estrogen deficiency, calcium and protein malnutrition [1]. Estrogen deficiency is considered as the major determinant of bone loss in postmenopausal women [2]. However, Kaplan and Hirsch [3] and Kin *et al.* [4] reported that osteoporosis may appear in aged men and women due to negative calcium balance.

Unfortunately, some of the clinically used osteoporotic therapy sometimes showed serious side effects. For example, alendronate (one of the bisphosphonate family) causes esophageal cancer,

gastrointestinal and osteonecrosis of the jaw on the long run [5]. Similarly, estrogen replacement therapy is accompanied by increased risk of breast, ovarian and endometrial cancer [6]. Therefore, it is still valuable to develop safer preventive medicine to suppress osteoporosis.

Nutrition is an important factor in the prevention and treatment of osteoporosis [7] and diets containing estrogen and calcium may contribute to maintaining the bone mass in postmenopausal women [8]. *Cicer arietinum* seed is one of the oldest and most widely planted legumes in the world [9]. For calcium supplement, the current work selects *Coelatura aegyptiaca* shell as it is one of the most common molluscan bivalves in Egypt as natural calcium source. *Coelatura aegyptiaca* belonging to Unionoidae and they are widely distributed along the River Nile from Assiut to Damietta [10].

Body weight gain of ovariectomized rats is considered one of the success factors of ovariectomy process, where the effect of ovariectomy was confirmed

by measuring the body weight [11]. Uterus, vagina and thymus are the primary target organs to estrogen and are considered sensitive parameters that used as evidence for the success of the surgical ovariectomy [12]. Therefore, Li *et al.* [13] reported that body weight and estrogen determinant organs weight (uterus, vagina and thymus) are considered as indicator for the quality of tested drug for treating bone loss.

The present study aims to investigate the effectiveness of *Cicer arietinum* seeds extract (CAE) and powder of *Coelatura aegyptiaca* shell (CES), either separately or concurrently, on ovariectomized rats induced osteoporosis. This study used ovariectomy model as it emulates estrogen depletion in postmenopausal women. Additionally, the ongoing study compares the effectiveness of the selected supplements against alendronate, one of the most common antiosteoporotic drugs.

MATERIALS AND METHODS

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 air-conditioned room at temperature of $23\pm 2^{\circ}\text{C}$ and 12 h light/dark cycle. They were feed 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, CUFS/F/PHY/0313) of Faculty of Science, Cairo University, Egypt. All the experimental procedures were carried out in accordance with international guidelines for care and use of laboratory animals.

Preparation of *Cicer arietinum* Extract: One gram of finely ground dry *Cicer arietinum* seeds was mixed with 4 ml of methanol, heated in a water bath at 60°C for 1 h while being shaken. The resulting extract was centrifuged at 10000 rpm, 5°C for 20 min. The resulting supernatant was filtered, concentrated and then lyophilized [14].

Preparation of *Coelatura aegyptiaca* Shell Powder: Freshwater mussel *Coelatura aegyptiaca* was collected and the soft flesh part was separated from the shell.

The shell powder was prepared according to the method described by [15]. The prepared *C. aegyptiaca* shell (CES) powder was stored until used.

Experimental Design: Forty two female albino rats were used. They were divided into 6 groups (7 rats/each group) as the following:

Group (I): Served as sham group (Vehicle). After one week of recovery from the sham surgery [11]. Rats of this group were administered orally distilled water for 10 weeks.

Group (II): Ovariectomized (OVX) rats orally administered distilled water daily for 10 weeks.

Group (III): OVX-rats treated orally with CAE daily for 10 weeks at a dose of 500 mg/kg b.wt/day. CAE dissolved in distilled water.

Group (IV): OVX-rats treated with aqueous suspension of CES powder (500 mg/ kg b.wt, orally) daily for 10 weeks.

Group (V): OVX-rats daily treated orally with both CAE and CES concurrently for 10 weeks.

Group (VI): OVX-rats treated with alendronate, suspended in distilled water, as a positive control one time/week (for 10 weeks) with a dose of 6.5 mg/ kg b.wt / week [16].

Body weights of all experimental groups were recorded weekly. After 10 weeks, rats were sacrificed and femurs, tibiae, thymus, vagina and uterus were excised and their surrounding tissues were removed. To reduce the individual body weight variation; the weights of femora, tibiae, thymus and vagina were recorded and weight of organ per 100 g of the body weight was calculated (relative weight of organ). Again, to determine the estrogenic activity of the extract under investigation (CAE), the ratio of uterine weight to body weight was calculated [17].

Statistical Analysis: Statistical analysis was carried out using SPSS v. 15 software. All data were expressed as means \pm standard error of mean (SEM). One way analysis of variance (ANOVA) followed by post hoc Duncan test used to compare between groups.

Table 1: Effect of CAE and CES administration singly or concurrently on body weight of rats.

Parameter	OVX treated with					
	Sham	Vehicle	CAE	CES	CAE + CES	Alendronate
Body weight gain (g)	25.285±3.037 ^a	50.571±9.534 ^b	33.000±2.708 ^{ac}	26.142±2.613 ^a	26.571±1.172 ^a	43.857±4.656 ^{bc}

• Values are mean±SEM (n= 7).

• Values with different superscript letters are significantly different (P < 0.05).

• OVX: ovariectomized rat; CAE: *Cicer arietinum* extract (500 mg/kg b.wt); CES: *Coelatura aegyptiaca* shell powder (500 mg/kg b.wt).

RESULTS AND DISCUSSION

Body weight growth of ovariectomized rats is considered one of the success factors of ovariectomy process, where the effect of ovariectomy was confirmed by measuring the body weight [11, 13]. Indeed, the current work revealed that the OVX group showed significant increase (P<0.05) in body weight than that of the sham group (Table 1). This result was found in accordance with the findings of Lim and Kim [18], Li *et al.* [13] and Ma *et al.* [19]. Folwarczna *et al.* [20] attributes this increase in body weight to estrogen deficiency caused by OVX. Additionally, the ongoing investigation interprets the body weight gain of OVX to one or both of the following reasons: (i) OVX rats gain weight due to ovariectomy-induced hyperphagia [21]. (ii) Other interpretation is that estrogen deficiency induces increase in adipose deposition and subsequently caused an increase in body weight [22]. Interestingly, overweight has been reported as a bone-protective factor for osteoporosis [23]. Again, the increase in body weight may be considered as a mechanism that provides an additional stimulus for bone regeneration and serving as a partial protection against the osteopenia which occurs in long bones [24]. Where adipose tissue considered one of the most important extragonadal sources of steroids and particularly estrogens. Wherever androstenedione converted into estrone in adipose cells through aromatization due to the specific expression of steroidogenic enzymes such as aromatase (estrogen synthase) in this tissue [25, 26]. Statistically, the increased body weight induced by ovariectomy was reversed significantly (P<0.05) after CAE (500 mg/kg b.wt) and CES (500 mg/kg b.wt) treatments, as compared to OVX rats. Moreover, the body mass gain of CAE and CES co-treated group was significantly (P<0.05) lowered than untreated OVX group. Similarly, regarding the body weight of OVX rats, Mvondo *et al.* [27] and Park *et al.* [27] investigated the ameliorative effect of *Erythrina lysistemon* (Fabaceae)

and many legumes, respectively. Szkudelska and Nogowski [28] and Cosma *et al.* [29] clarified that genistein, present in many legumes, decreased body weight gain by increasing lipolysis and decreasing lipogenesis via the ER α signaling pathway. This confirms that CAE mimic the endogenous estrogen effect by inducing a significant body weight loss and this proves the effectiveness of this extract on ER α . Moreover, the decreased body weight gain of CAE relative to OVX may be attributed to its tannin content which reduces the food consumption as reported by Rangrez *et al.* [30]. Regarding calcium supplement treatment, the current study revealed that CES significantly suppressed the body weight gain of OVX and this is in consonance with the result of Breitman *et al.* [8] who used calcium supplement in diet. A possible reason is that calcium may have regulated body weight gain. Zemel [31] clarified that calcium inhibits lipogenesis, increases lipolysis and increases thermogenesis leading to a net reduction in fat mass. The present work suggested that rats treated with CAE and CES concurrently lowered the final body weight than rats in the untreated OVX group and this finding was found in line with Breitman *et al.* [8]. Conversely, OVX induced body weight gain not affected by alendronate administration and this is in consonance with Ho *et al.* [32].

The present study revealed that removal of ovaries caused significant decrease (P<0.05) in the absolute and relative femoral weight values, as compared to sham femoral weight (Table 2). Ovariectomy process caused a significant decrease (P< 0.05) in the tibial weight value either absolute or relative to the body weight, as compared to sham value (Table 3). Administration of CAE (500 mg/kg b.wt) caused a marked increase in the tibial weight values, but this increase was found statistically significant (P<0.05) only in case of the absolute value, as compared to OVX value. Table 3 shows an increase in the tibial weight values (absolute or relative) of rats after oral administration of CES (500 mg/kg b.wt), but this increase

Table 2: Effect of CAE and CES administration singly or concurrently on absolute and relative femoral weight of rats

Parameters	OVX treated with					
	Sham	Vehicle	CAE	CES	CAE + CES	Alendronate
Femoral weight Absolute (mg)	942.842±46.977 ^a	801.985±43.440 ^b	1207.828±58.644 ^d	924.156±28.096 ^a	1080.970±17.051 ^c	963.742±35.469 ^a
Relative (mg/100 g b.wt)	839.487±24.958 ^a	601.822±25.5 ^b	785.897±42.238 ^{ac}	823.411±21.995 ^a	741.057±25.422 ^{ac}	721.797±25.463 ^c

► Values are mean±SEM (n= 7).

► Values with different superscript letters are significantly different (P < 0.05).

► OVX: ovariectomized rat; CAE: *Cicer arietinum* extract (500 mg/kg b.wt); CES: *Coelatura aegyptiaca* shell powder (500 mg/kg b.wt).

Table 3: Effect of CAE and CES administration singly or concurrently on absolute and relative tibial weight of rats

Parameters	OVX treated with					
	Sham	Vehicle	CAE	CES	CAE +CES	Alendronate
Tibial weight Absolute (mg)	839.657±36.532 ^a	668.042±22.323 ^b	827.271±56.908 ^a	673.485±13.817 ^b	818.114±24.087 ^a	708.385±29.741 ^{bc}
Relative (mg/100 g b.wt)	753.641±42.225 ^a	492.75±23.521 ^b	567.721±31.663 ^{bc}	612.932±18.315 ^c	540.86±20.813 ^{bc}	537.709±35.867 ^{bc}

► Values are mean±SEM (n= 7).

► Values with different superscript letters are significantly different (P < 0.05).

► OVX: ovariectomized rat; CAE: *Cicer arietinum* extract (500 mg/kg b.wt); CES: *Coelatura aegyptiaca* shell powder (500 mg/kg b.wt).

was significant (P<0.05) only in case of the relative value, as compared to OVX value. Conversely, concomitant administration of CAE and CES increased the absolute tibial weight value significantly (P<0.05) versus to OVX value. Statistically, a non-significant change was recorded in the tibial weight value subsequent to alendronate administration, as compared to the corresponding OVX value (Table 3). This result was found in agreement with the results of Folwarczna *et al.* [20] and Wahba and Al-Zahrany [33]. Occhiuto *et al.* [34] and Masuda *et al.* [35] mentioned that ovariectomized rats developed bone changes similar to those seen in osteoporotic women as indicated by a decrease in femur weight. The bone weight loss is thought to have resulted from bone resorption enhanced by estrogen deficiency. On the contrary, supplementation with CAE or/and CES increased the femoral and tibial weight, as compared with OVX rats. These findings were found in consonance with the findings of Potu *et al.* [36], Seo *et al.* [37] and Wahba and Al-Zahrany [33]. The current study suggested that the administration of CAE or /and CES may accelerate bone formation that will cause an increase in bone weight and this interpretation is in line with Masuda *et al.* [35]. Daidzein (one of phytoestrogen compounds) has an important role in stimulation as well as acceleration of

bone formation [38]. In addition, for calcium supplement treatment; there is evidence that dietary intake of adequate calcium and phosphorus is required for bone growth through bone formation and mineralization [39]. Furthermore, the current study recorded that significant increment (p< 0.05) of bone weight (femurs and tibias) was occurred due to alendronate treatment (Table 2). This finding is similar with the finding of Seo *et al.* [37]. But, this outcome is against the facts that alendronate inhibit bone resorption and fail to restore lost bone mass [40]. The ongoing study attribute this to duration difference of alendronate treatment, wherever McClung *et al.* [41] disclosed that after 1 month of alendronate treatment; it suppress bone resorption while inhibition of formation occurred after 3 months.

The bilaterally ovariectomized rats revealed a significant decrease (P< 0.05) in the uterus weight values (absolute and relative), as compared to the non-ovariectomized control (Fig. 1). Oral administration of CAE daily for ten weeks to the OVX rats increased significantly (P<0.05) the uterus weight, as compared to untreated OVX rats. Statistically, administration of CES (500 mg/kg b.wt) for ten weeks to the OVX rats did not significantly affect the mass of the uterus, as compared to

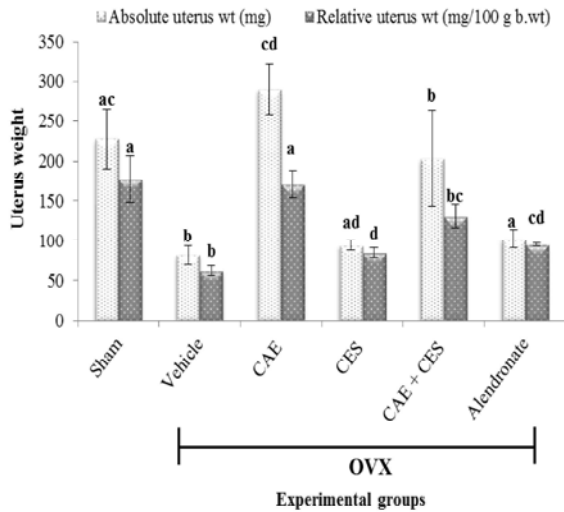


Fig. 1: Effect of CAE and CES administration singly or concurrently on absolute and relative uterus weights of rats.

- ▶ Values are mean±SEM (n= 7).
- ▶ Values with different superscript letters are significantly different (P < 0.05).
- ▶ OVX: ovariectomized rat; CAE: *Cicer arietinum* extract (500 mg/kg b.wt); CES: *Coelatura aegyptiaca* shell powder (500 mg/kg b.wt).

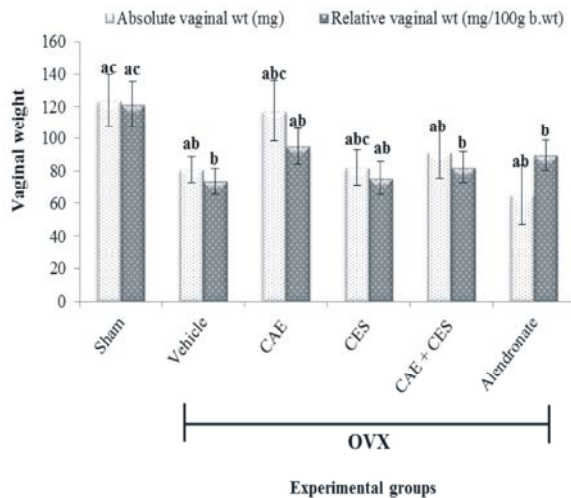


Fig. 2: Effect of CAE and CES administration singly or concurrently on absolute and relative vaginal weights of rats.

- ▶ Values are mean±SEM (n= 7).
- ▶ Values with different superscript letters are significantly different (P < 0.05).
- ▶ OVX: ovariectomized rat; CAE: *Cicer arietinum* extract (500 mg/kg b.wt); CES: *Coelatura aegyptiaca* shell powder (500 mg/kg b.wt).

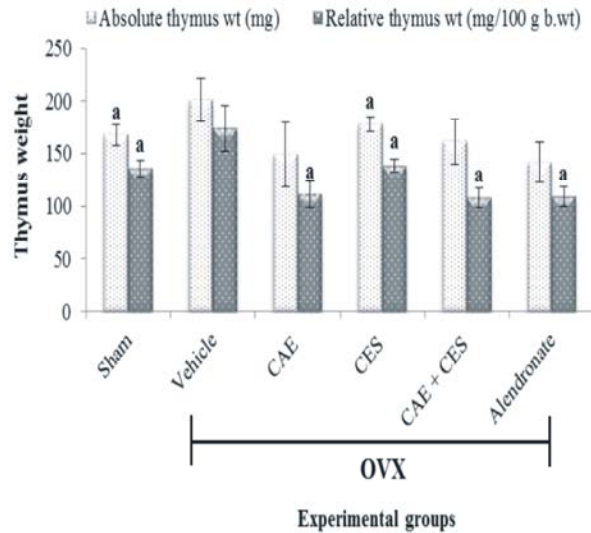


Fig. 3: Effect of CAE and CES administration singly or concurrently on absolute and relative thymus weights of rats.

- ▶ Values are mean±SEM (n= 7).
- ▶ Values with different superscript letters are significantly different (P < 0.05).
- ▶ OVX: ovariectomized rat; CAE: *Cicer arietinum* extract (500 mg/kg b.wt); CES: *Coelatura aegyptiaca* shell powder (500 mg/kg b.wt).

OVX group. Again, concurrent administration of CAE and CES counteracted the decrease of the uterus mass caused by OVX, since, a significant increase (P < 0.05) was recorded, as compared to OVX rats (Fig. 1). On the other hand, non-significant change was disclosed subsequent to alendronate administration in comparison to the OVX value.

Figure 2 demonstrates that the relative vaginal weight decreased significantly (P < 0.05) in the OVX rats, as compared to the sham-operated rats. From the statistical point of view, this decrease in vaginal weight of the OVX-rats did not affected by the administration of CAE, CES and their concomitance when compared to the OVX values. Again, a non-significant change was observed in the vaginal weight after alendronate administration, relative to the OVX group.

A significant increase (P < 0.05) was recorded in the relative thymus weight value of OVX rats (Fig. 3). It is interesting enough to noticed that the treatment with CAE or CES either singly or concurrently restored the relative thymus weight value significantly (P < 0.05), as compared to OVX values (Fig. 3).

Additionally, a significant decrease ($P < 0.05$) was disclosed in the relative thymus weight value subsequent to administration of alendronate, as compared to sham control group.

Uterus, vagina and thymus are the primary target organs to estrogen and are considered sensitive parameters that used as evidence for the success of the surgical ovariectomy [12]. With respect to uterus weight; OVX caused a female hormone deficiency, thereby results in atrophy of the uterus (in this study) as demonstrated by significant decrease recorded in the uterus weight. This finding was found in accordance with some previous investigators [26, 42, 43]. In consistent with the findings of Zhang *et al.* [12]; Mvondo *et al.* [26] and Kawakita *et al.* [44], the present study recorded that the CAE administration caused estrogenic effect by increasing the uterus weight of OVX rats significantly, but it was still lower than that of the sham rats. Additionally, Zhang *et al.* [45] reported that the uterus express ER α and exert stimulatory effect when bind to estrogen, thereby, the current study suggests that CAE may bind with estrogen receptor and thus increase the uterus weight. Regarding the effect of calcium supplement, a non-significant change was recorded subsequent to CES administration. This finding was found partially in agreement with Breitman *et al.* [8] and this finding may confirm the beneficial effect of CES in reducing the risk of diseases associated with estrogen replacement as Zhang *et al.* [46] concluded on *Achyranthes bidentata*. The current finding revealed that alendronate has no effect on the uterus weight, as compared to OVX and this is in agreement with the findings of Bitto *et al.* [47].

With respect to vaginal weight, ovariectomy brought about a strikingly decrease in vaginal weight in rats, as compared to that of sham group. This may be attributed to estrogen deficiency [48,49]. Westwood [50] reported that during the *in vivo* study, the loss of ovarian estrogen following surgical menopause caused marked vaginal epithelium thinness. Moreover, non-significant change was observed due to oral administration of CAE and CES singly or concurrently, as compared to OVX group.

These results were found similar with the findings of Ko *et al.* [48] and Yang *et al.* [49]. The present finding revealed that an insignificant effect was detected on the vaginal weight after alendronate administration and this

was found in consistence with the findings of Ho *et al.* [32] and Ko *et al.* [48].

The present study revealed that bilateral ovariectomy in rats increased thymus weight in comparison with the non-ovariectomized ones. This finding was found in consistent with some investigators [20, 51, 52]. D'Amelio *et al.* [53] revealed that in mice and human thymopoiesis is important soon after the fall in sexual hormone levels, where orchietomy or ovariectomy enhanced regeneration of the aged mouse and human thymus. It seems that the recorded increase in thymus weight of OVX rat may be due to estrogen deficiency and/or thymus cell regeneration [53, 54]. Rats treated with CAE or/and CES reduced the thymus weight significantly, as compared to OVX rats. This finding was found in agreement with Shih *et al.* [55] who disclosed ameliorative effects of *Vaccaria segetalis* extract on osteopenia in ovariectomized rats. It was reported that estradiol initiates thymus atrophy by inhibiting thymocyte development at multiple stages [56]. Additionally, Yellayi *et al.* [57] declared that genistein reduce hypertrophy of thymus weight resulted from OVX. The present finding revealed that alendronate reduced the thymus mass significantly; relative to OVX rats and this agree with Funayama *et al.* [58] and Yu *et al.* [59]. The present work suggests that alendronate may be impaired the development of the thymocytes as Milhaud *et al.* [60] who worked on clodronate, one of bisphosphonate family.

In conclusion, the results of the present study revealed that the combination of CAE and CES may be effective in treating osteoporosis. This demonstrated by their ameliorative effect on estrogen dependent organs (uterus, vagina and thymus). Therefore, the present study provides insight to further investigation to understanding the potential positive impact of CAE-CES interactions. Additionally, further studies needed to analyze the CAE and CES contents that may contribute in the mechanism of action against osteoporosis.

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